Inflammatory signalling pathways in cystic fibrosis airway epithelial cells

Lavelle, Michaela J.

Award date:
2002

Awarding institution:
University of Bath

INFLAMMATORY SIGNALLING PATHWAYS IN CYSTIC FIBROSIS AIRWAY EPITHELIAL CELLS

Submitted by Michaela J. Lavelle

for the degree of PhD

of the University of Bath

2002

COPYRIGHT

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

CONTENTS

ACKNOWLEDGEMENTS

Firstly I would like to thank my two supervisors, Dr. Malcolm Watson and Dr. Anthony Smith for all their supervision, help and guidance throughout the course of this study. I will always be very grateful to them for giving me the opportunity to get back into research.

Many people have given me their support to help finish this work, but I would particularly like to thank Dr. Helen Brown for her advice and help with cell culture and immunoblot analysis, Jane Leithead for her instructions in ELISA techniques and Dr. Stephen Ward for his invaluable guidance with the cell signalling aspects of this project. I would also like to give a special mention to Victoria Reidy, Stephanie Williams, Mona Bustami, Gillian Whittaker and Clare Baker who have helped to make my time at Bath very special and who have provided continued support and encouragement along the way. Thank you for your friendship and I hope we will remain in contact.

Most importantly I would like to thank my husband Sean, as I could not have completed this thesis without his love, patience and understanding. He has been a huge motivator for me and has always believed that I could do this, even when I did not! Thanks must also go to my mum and the rest of my family and friends who have always provided encouragement and had to put up with me ensconcing myself in my study for weeks on end during the last few months.

Finally, I would also like to thank the Medical Research Council and Novartis for their generous financial support.

ABSTRACT

It has been reported that CF airway epithelium is deficient in inducible nitric oxide synthase (iNOS) activity. It has also been proposed that CF airway epithelial cells are primed to produce high levels of the neutrophil attracting chemokine interleukin-8 (IL-8) in response to infection. Recently, activation of phosphatidylinositol 3-kinase (PI3K) has been linked to the inhibition of iNOS transcription in epithelial cells. In this study the potential of human CF and non-CF airway epithelial cells to produce NO and IL-8 was examined and the possible role of the PI3K signalling pathway in the regulation of these inflammatory mediators was investigated.

The airway epithelial cell lines 9HTEo$^-$, ΣCFTE29o$^-$, 9HTEo$^-$/pCEP#2 and 9HTEo$^-$/pCEPRF were found to produce basal levels of NO but these were not increased by proinflammatory cytokine stimulation. This lack of response was not a consequence of substrate limitation or competition. Proinflammatory cytokines were found to induce IL-8 expression in all four cell lines. The levels of IL-8 produced by the CF phenotype cell line, 9HTEo$^-$/pCEPRF in response to proinflammatory stimuli were significantly higher than those produced by the 9HTEo-/pCEP#2 cell line, a non-CF genetically matched control. This difference may be a consequence of impaired cAMP-dependent chloride ion transport. No link was found between the regulation of IL-8 synthesis and the PI3K signalling pathway.

Interleukin-13 (IL-13) was shown to activate PI3K and lead to the phosphorylation of protein kinase B (PKB) and glycogen synthase kinase-3 (GSK3) in human airway epithelial cells. The ERK1/2 and JNK MAP kinase pathways and the PKC pathway were shown to play a larger role in GSK3 phosphorylation in the CF phenotype ΣCFTE29o$^-$ cell line than the non-CF 9HTEo$^-$ cell line. These findings could have implications for the control of the many cellular processes that are regulated by GSK3, such as the regulation of inflammatory gene transcription and the control of cell survival pathways.

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ABC	ATP binding cassette
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AP-1	Activator protein-1
AP-2	Activator protein-2
AP-3	Activator protein-3
ASF	Airway surface fluid
ATP	Adenosine trisphosphate
BAD	Bcl-2 antagonist of cell death
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BH_4	Tetrahydrobiopterin
BSA	Bovine serum albumin
cAMP	Cyclic adenosine-5'-monophosphate
CBP/p300	CREB binding protein/p300
CCR	CC chemokine receptor
C/EBP	CCAAT/enhancer binding protein sequence
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
cGMP	Cyclic guanosine-5-'monophosphate
cNOS	Constitutive nitric oxide synthase
CREB	c-AMP response element binding protein
CXCR	CXC chemokine receptor

DAN	2,3-diaminonapthalene
ΔF508	Omission of phenylalanine residue at amino acid position 508
DLG	*Drosophila melanogaster* discs large protein
DMEM	Dublecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
ENaC	Amiloride-sensitive epithelial sodium channel
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal regulated kinase
FAD	Flavine dinucleotide
FBS	Foetal bovine serum
FCS	Foetal calf serum
FKHLR1	Forkhead transcription factor
FMN	Flavine mononucleotide
GSK3	Glycogen synthase kinase-3
[^{3}H]	Hydrogen radiolabel
hBD2	Human beta defensin 2
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HPLC	High pressure liquid chromatography
IFNγ	Interferon gamma
IκB	Inhibitory kappa B protein

IKK	Inhibitory kappa B kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF-1	Interferon regulatory factor-1
JNK	c-jun amino terminal kinase
LPS	Bacterial lipopolysaccharide
MAGI2	Membrane-associated guanylate kinase inverted-2
MAGI3	Membrane-associated guanylate kinase inverted-3
MAGUK	Membrane-associated guanylate cyclase
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MEK	Mitogen-activated protein kinase kinase
MEM	Eagle's minimum essential medium
MMAC1	Mutated in multiple advanced cancers-1
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NBD	Nucleotide binding domain
NF-IL-6	Nuclear factor of interleukin-6
NF-κB	Nuclear factor kappa B
NHERF/EBP50	Sodium ion / hydrogen ion exchanger regulatory factor or ezrin-radixin-moesin binding phosphoprotein of 50 kDa
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NP-40	Nonidet P-40

OD	Optical density
OPD	O-phenylene dihydrochloride
[^{32}P]	Phosphate radiolabel
PAC	PTEN-associated complex
PARS	Poly-ADP ribose synthase enzyme
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PDK1	Phosphoinositide-dependent kinase-1
PDZ	PSD-95, DLG, ZO-1 domain
PH	Pleckstrin homology domain
PI	Phosphoinositide lipid
PI3K	Phosphatidylinositol 3-kinase
PKA	cAMP-dependent protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKR	Double-stranded RNA-activated protein kinase
PMN	Polymorphonuclear leukocyte
PMSF	Phenylmethylsulphonyl fluoride
PNR	*Pseudomonas* nitrite reductase
PP2A	Protein phosphatase 2A
PP2C	Protein phosphatase 2C
PSD-95	95 kDa protein involved in signalling at the post-synaptic density
PtdIns-3-P	Phosphatidylinositol-3-monophosphate
PtdIns-3,4-P_2	Phosphatidylinositol-3,4-bisphosphate
PtdIns-3,5-P_2	Phosphatidylinositol-3,5-bisphosphate
PtdIns-3,4,5-P_3	Phosphatidylinositol-3,4,5-trisphosphate

PTEN	Phosphatase and tensin homologue deleted on chromosome ten
R	Regulatory domain
RANTES	Regulated on activation, normal T cell expressed and secreted
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SH2	Src-homology-2 domain
STAT-1	Signal transducer and activator of transcription-1
TBS	TRIS buffered saline
TEMED	N,N,N',N', tetramethylene-diamine
TEP1	Transforming growth factor beta-regulated and epithelial-cell-enriched phosphatase
TGFβ	Transforming growth factor beta
Th2	T helper 2
TLC	Thin-layer chromatography
TNFα	Tumour necrosis factor alpha
Tween-20	Polyoxyethylenesorbitan monolaurate
ZO-1	Zonula occludens 1 protein

1 INTRODUCTION

1.1 AN OVERVIEW OF CYSTIC FIBROSIS

Cystic fibrosis (CF) is an autosomal recessive genetic disorder affecting children and young adults (Boat *et al.*, 1989). It is the most common life-threatening genetic trait in the Caucasian population. In Northern Europe the heterozygote frequency is about 1:25 and the disease frequency is approximately 1:2500 live births. The dominant clinical feature of CF is the obstruction of airways by thick, sticky mucus and subsequent infection, primarily by *Pseudomonas aeruginosa.* As the disease progresses some patients may also become infected with *Burkholderia cepacia* (Armstrong *et al.*, 1995; Bauernfeind *et al.*, 1997). Once these pathogens are established it is extremely difficult to eradicate them and they cause rapid deterioration of lung function. Although relatively uncommon, *B. cepacia* is particularly difficult to control as it is resistant to most antimicrobial agents. Chronic inflammation results from the presence of persistent infection and this contributes to the obstructive lung disease and tissue destruction. The final consequence is often respiratory failure. As a result, lung infection is the major cause of mortality for cystic fibrosis patients.

The gastro-intestinal tract is also affected in CF. Approximately 85% of patients show pancreatic insufficiency as a result of mucus obstruction of the pancreatic ducts and destruction of exocrine function. In addition, 5-10% of newborns with CF suffer from an intestinal obstruction termed meconium ileus. This obstruction is caused by incomplete digestion of intraluminal contents *in utero* and is generally ascribed to failure of pancreatic enzyme secretion and a decrease in fluid secretion. In adults with CF, infertility is almost universal in males and common in females (Boat *et al.*, 1989).

At present the management of CF includes chest percussion to clear mucus from the airways and improve lung function, the use of antimicrobials to deal with lung infection, pancreatic enzyme replacement to aid digestion and close attention to diet (Boat *et al.*, 1989). As a result of these combined therapies survival has improved steadily over the last four decades, with the median survival of patients born in 1990 now being approximately 40 years of age (Jaffe, 2001)

1.1.1 The Discovery of the CF Gene

CF was first described as a distinct clinical entity in the late 1930s. In 1983 Paul Quinton showed that chloride uptake by absorptive sweat duct epithelium was abolished in CF. This characteristic is now used as a diagnostic tool for the disease. Shortly afterwards cyclic AMP-dependent transport of chloride ions across airway epithelia was also shown to be greatly reduced in CF and the defect was localised to the apical membrane (Stutts *et al.*, 1985; Widdicombe *et al.*, 1985; Yankaskas *et al.*, 1985[b]). Studies suggested that this defect was probably due to the failure of an outwardly rectifying anion channel to respond to phosphorylation by a cyclic AMP-dependent protein kinase (Welsh, 1986; Frizzell *et al.*, 1986; Schoumacher *et al.*, 1987; Li *et al.*, 1988). However, this information alone was not sufficient to identify directly the protein product of the gene that is defective in this disease.

In 1989 researchers successfully identified the defective gene responsible for CF; it was found to be on chromosome 7 (Kerem *et al.*, 1989; Riordan *et al.*, 1989). Analysis of the predicted gene product revealed a transmembrane protein that had the structure of a regulated ion channel. The protein was termed the cystic fibrosis transmembrane

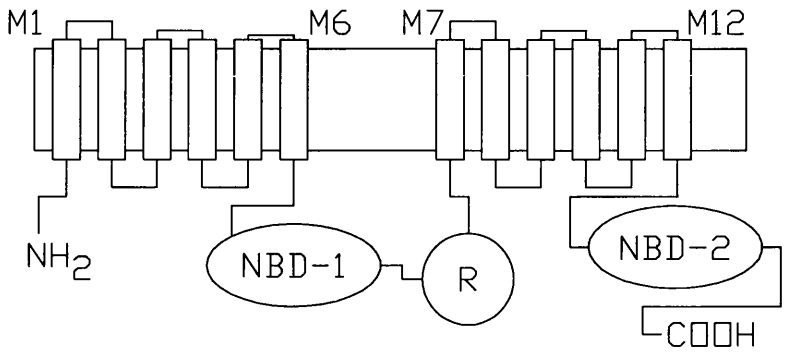
conductance regulator (CFTR) because of the link between abnormal chloride ion transport and the CF defect.

1.1.2 Structure and Function of the Cystic Fibrosis Transmembrane Conductance Regulator

Functional CFTR is expressed on the apical surface of secretory epithelial cells. It is a member of the ATP-binding cassette (ABC) family. Members of this family exhibit a range of functions, including ATP-dependent transmembrane transport of large molecules, regulation of other membrane transporters and ion conductance (Wine, 1999). CFTR shows at least two of these properties, being a chloride ion channel and a regulator of other membrane proteins. For example, CFTR negatively regulates the amiloride-sensitive epithelial sodium channel (ENaC) (Knowles *et al.*, 1993). Many regulatory roles have been proposed for CFTR, however it is its role in ion transport that has influenced most current hypotheses of CF disease.

CFTR is a 1480 amino acid glycosylated membrane glycoprotein. It is composed of two homologous halves which each have six membrane spanning segments and one nucleotide binding domain (NBD) (Fig 1). A cytoplasmic regulatory domain (R), containing a number of consensus phosphorylation sites, links the two homologous halves. Residues from the twelve membrane spanning segments form the channel lining (reviewed in Akabas, 2000).

Figure 1 The transmembrane topology of CFTR (adapted from Akabas, 2000).



Abbreviations: M1-M12, membrane spanning segments; NH_2, N-terminal domain; COOH, C-terminal domain; NBD-1, nucleotide binding domain-1; NBD-2, nucleotide binding domain-2; R, cytoplasmic regulatory domain

Two separate processes control the gating of CFTR. Firstly, serine residues in the regulatory domain are phosphorylated by a cAMP-dependent protein kinase (PKA). The more extensive this phosphorylation, the greater the probability that the channel will open. Secondly, the nucleotide binding domains bind and hydrolyse ATP (Cheng *et al.*, 1991). Researchers have traditionally proposed that unphosphorylated R domain inhibits the channel by physically blocking the pore and that phosphorylation relieves this inhibition (Collins, 1992; *Rich et al.*, 1993). However more recently it has been demonstrated that unphosphorylated R domain does not 'act like a plug' but that phosphorylation of R stimulates interaction between the NBDs and ATP (Winter *et al.*, 1997). The hydrolysis of ATP by the NBDs causes the channel to open and anions flow through it along an electrochemical gradient. The R domain can be

dephosphorylated by the protein phosphatases PP2C and PP2A and this closes the channel (Vankeerberghen *et al.*, 2001).

CFTR also directly interacts with other proteins. Its cytoplasmic N-terminal domain has a direct interaction with syntaxin 1A. This protein is known to inhibit channel activity. At its C-terminus it interacts with a family of PDZ domain containing proteins (Akabas, 2000). The term PDZ derives from the first three proteins in which these domains were identified: PSD-95 (a 95 kDa protein involved in signalling at the post-synaptic density), DLG (the *Drosophila melanogaster* Discs Large protein) and ZO-1 (the zonula occludens 1 protein involved in the maintenance of epithelial polarity) (reviewed in Harris & Lim, 2001). *In vivo* these domains are thought to be involved primarily in the organization of multiprotein complexes that function in signalling, as well as in the establishment and maintenance of cell polarity. They particularly recognise specific C-terminal motifs called PDZ interacting domains, in the cytoplasmic tails of transmembrane receptors and channels (Kornau *et al.*, 1995; Niethammer *et al.*, 1996). It has been demonstrated that a C-terminal PDZ interacting domain is required for the polarization of CFTR to the apical plasma membrane in airway and kidney epithelial cells, although the mechanism behind this directed polarized expression is not known (Moyer *et al.*, 1999; Moyer *et al.*, 2000; Milewski *et al.*, 2001). Recent evidence suggests that PDZ domain containing proteins may also have direct affects on CFTR activity as well as on membrane localization. Two studies have shown that the Na^+/H^+ exchanger regulatory factor, or ezrin-radixin-moesin binding phosphoprotein of 50 kDa (NHERF/EBP50) can directly increase conductance through the CFTR channel via binding of its PDZ domains to the C-terminal tail of CFTR (Wang *et al.*, 2000; Raghuram *et al.*, 2001). However, it is not clear exactly how this interaction with NHERF/EBP50 causes a change in conductance.

1.1.3 The CF Gene Defect

There are a range of mutations which may lead to CF (reviewed by Zeitlin, 1999).

1. **Class I mutations** - These lead to the synthesis of unstable/truncated CFTR mRNA, usually due to premature stop codons. The result is a total absence of CFTR protein. The disease phenotype of this class is often severe.
2. **Class II mutations** – These are responsible for CF in approximately 70% of patients. The most frequent mutation, ΔF508 is in this class. The ΔF508 mutation corresponds to a specific deletion of three base pairs, which results in the omission of a phenylalanine residue at amino acid position 508 in the centre of the first NBD of the CF gene product (Kerem *et al.*, 1989; Riordan *et al.*, 1989). The established view is that the protein is translated but fails to escape the endoplasmic reticulum. It is thought that misfolding of the protein targets it for early proteolysis and as a result little or no CFTR reaches the plasma membrane. However, work by Kalin *et al.* (1999) on epithelial tissue from ΔF508 homozygous CF patients, demonstrated an absence of CFTR in sweat duct epithelium, but the presence of CFTR in respiratory and intestinal epithelium. This suggests that expression of ΔF508 CFTR shows tissue specific variation. The disease phenotype of this class is often severe.
3. **Class III mutations** – These disrupt activation of CFTR at the plasma membrane (biosynthesis, trafficking and processing are undisturbed). The channel is probably defective with respect to ATP binding and hydrolysis or R domain phosphorylation. This can also result in a severe disease phenotype.
4. **Class IV mutations** – The mutations are in the membrane spanning regions which form the chloride ion channel. These mutations may affect the rate of

chloride ion flow or channel open time. This class confers a mild disease phenotype.

5. **Class V mutations** – These lead to a decrease in the level of normal CFTR by alterations to the promoter. If the reduction of CFTR is greater than 10% this will lead to disease.

The mutations can act in combination. For example the deletion of the codon for phenylalanine 508 can act alongside a reduced channel opening time. A small set of these mutations may confer residual pancreatic exocrine function in a subgroup of patients who are pancreatic sufficient.

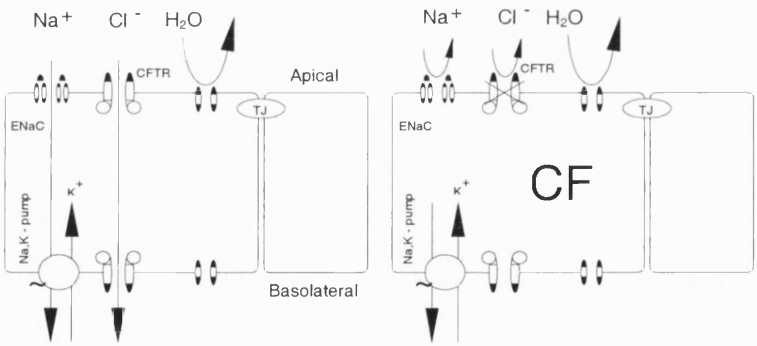
1.1.4 The Role of CFTR in CF lung Disease

Two main hypotheses exist to explain the effect of mutated CFTR on the function of lung epithelia. They both consider the role of CFTR in salt absorption to be of primary importance.

1. **The high salt hypothesis. Smith *et al.*, 1996** - This hypothesis emphasizes the function of CFTR as an anion channel. According to Smith and colleagues, missing or defective CFTR leads to a decrease in transepithelial chloride conductance. By analogy with CF sweat duct epithelium (Quinton, 1983) they propose the consequence of this is reduced movement of sodium through amiloride sensitive sodium channels (Fig. 2). The overall result is an increased salt concentration in the airway surface fluid (ASF) of the CF lung. This high salt concentration could interfere with natural antibiotics in the ASF such as defensins and lysozyme. Smith *et al.* suggest that normal airways, like sweat

ducts, reabsorb salt in an excess of water from the ASF, therefore producing a low salt content in the ASF which does not affect the action of antimicrobial agents. Zabner *et al.* (1998), have provided evidence to support this hypothesis by demonstrating that apical fluid covering CF cells is approximately twice as salty as normal in their culture system. However, it is worth noting that *in vitro* defensin activity is negligible above 50 mM salt and one study has found a chloride salt concentration of 170 ± 79 mM in bronchial ASF from CF patients versus 85 ± 54 mM in control subjects (Gilljam *et al.*, 1989). Findings such as these cast doubt on the importance of such changes in salt concentration and/or the accuracy of ASF salt measurements.

Figure 2 The high salt hypothesis (adapted from Wine, 1999).

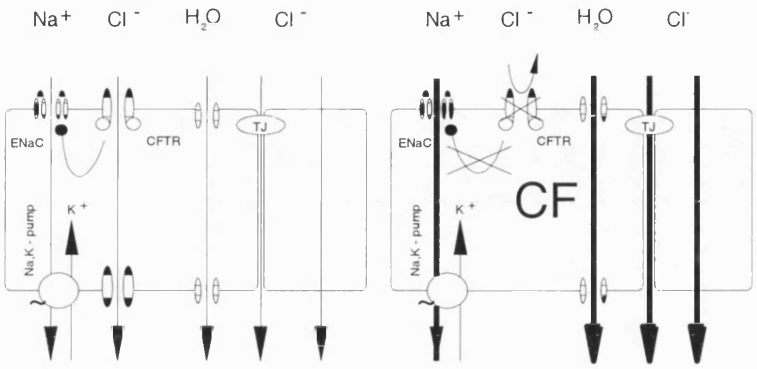


Abbreviations: ENaC, epithelial sodium channel; CFTR, cystic fibrosis transmembrane conductance regulator; TJ, tight junction; Na,K pump, sodium, potassium pump.

Key features of this model are: the central importance of CFTRs channel role, the lack of alternative non-CFTR pathways for the transport of chloride ions and no specific role for the inhibition of ENaC by CFTR.

2. **The low volume hypothesis. Matsui *et al.*, 1998** – This hypothesis is based on CFTR regulation of the amiloride sensitive epithelial sodium channel (ENaC). Matsui and colleagues found no difference in the salt concentration of the ASF from normal and CF airway cultures. However they did find differences in the volume of the ASF from these cultures; the liquid layer in CF epithelial cell cultures was reduced in volume compared with that from normal airway cultures. The researchers suggest that CFTR mutations eliminate the inhibition by CFTR on ENaC. This leads to increased sodium absorption, via ENaC, from the apical surface. Chloride ions follow via a non-CFTR shunt pathway. As a consequence water flows transcellularly from the apical fluid (Fig. 3). The overall result is ASF containing a decreased volume of water (i.e. more viscous mucus). This would compromise mucociliary clearance and promote infection.

Figure 3 The low volume hypothesis (adapted from Wine, 1999).



Abbreviations: ENaC, epithelial sodium channel; CFTR, cystic fibrosis transmembrane conductance regulator; TJ, tight junction; Na,K pump, sodium, potassium pump.

Key features of this model are: the parallel pathway for chloride ion conductance and the inhibition of ENaC by CFTR.

These two hypotheses are different. It is possible that each group is accurately measuring the properties of their cell cultures, but that these properties differ (Wine, 1999). Cultured epithelial cells exhibit different degrees of CFTR expression, water permeability and shunt resistance. These features can affect epithelial transport properties and the results of CFTR malfunction. *In vivo* experiments are needed to determine the composition of ASF in actual human airways. However, it is technically very challenging to perform such experiments.

Whilst these theories may account for some of the symptoms of CF, they do not easily explain many of the lung manifestations that are responsible for approximately 90% of patient deaths. CF airway disease is characterised by long lasting bacterial infections with persistent local and systemic inflammation. How does the exaggerated inflammatory response relate to the CF defect?

1.1.5 Airway Host Defence and CF

The epithelium of the mammalian airway is in close contact with the outside world and this provides a vast opportunity for infection. However the incidence of infection in a normal, healthy lung is very low. It has become evident in recent years that the ciliated epithelium lining the airway prevents colonization by inhaled bacteria in three ways. Firstly, physical processes such as induced turbulent flow of inspired air, coughing, ciliary clearance and epithelial cell shedding act to remove much of the inhaled material. Secondly, constitutively expressed and inducible chemical defences serve to provide a broad-spectrum of antimicrobial agents. Finally, phagocytic cells are recruited to the site of infection and an inflammatory response is mobilised (reviewed by Bevins, 1999).

In the CF lung the presence of thick, sticky mucus has a detrimental effect on many of the physical processes forming the initial line of defence in the airway. Inhaled organisms therefore gain a foothold and infection is easily established. This problem is compounded by the failure of CF airway epithelial cells to ingest and subsequently inactivate bacteria and the apparent increased binding of certain bacteria to the surface of these cells. Both of these defects have been directly linked to mutated CFTR (Pier *et al.*, 1996; Zar *et al.*, 1995).

Examinations of the constitutive antimicrobial agents found in the mucus layer covering healthy airway epithelium have identified a wide range of peptides, proteins and organic molecules. Two of the most important of these host defence molecules are lysozyme and lactoferrin, which are both broad-spectrum antibiotic proteins (Travis *et al.*, 1999). They have different modes of action; whereas lysozyme causes lysis of bacterial cells, lactoferrin brings about their agglutination (Cole *et al.*, 1999; Harbitz *et al.*, 1984). Many other potential antimicrobial agents have also been described including secretory leukoprotease inhibitor, uric acid, peroxidase, aminopeptidase, statherin, secretory phospholipase A2 and α/β-defensins (Kaliner, 1991; Cole *et al.*, 1999; Diamond & Bevins, 1998). These all have a wide and diverse range of actions. It is the activities of these antimicrobials, which may be affected if salt concentrations are elevated in the CF lung (Smith *et al.*, 1996).

Two complementary inducible defence mechanisms have also been observed in the response of healthy airway epithelium to infection (reviewed by Diamond *et al.*, 2000). The first mechanism is the increased production of antimicrobial agents and the second mechanism is the induction of a signaling network to recruit phagocytic cells to the infectious site. Airway epithelial cells will upregulate the expression of many antimicrobials in response to numerous infectious and inflammatory agents, including bacterial lipopolysaccharide (LPS) and the proinflammatory cytokines tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β) and interferon gamma (IFNγ). Activated alveolar macrophages are a primary source of proinflammatory cytokines in the infected airway. Examples of antimicrobials that are induced in this manner include human β-defensin 2 (hBD2), mucins and reactive nitrogen species such as nitric oxide (Diamond *et al.*, 2000). Together these antimicrobial agents act to prevent further

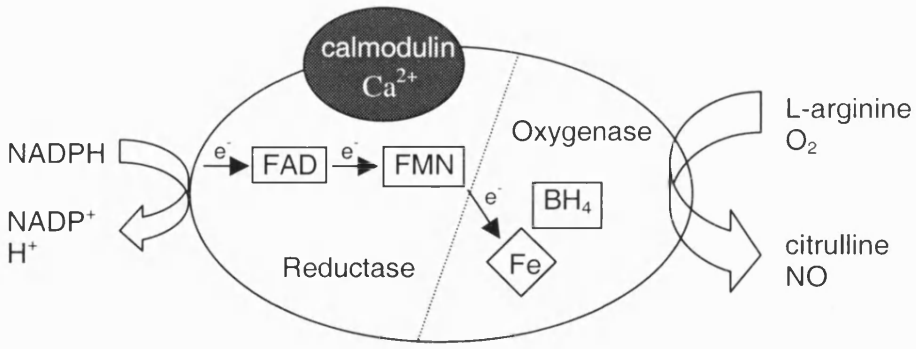
colonization by infecting microbes. Alveolar macrophages are also capable of generating numerous other inflammatory mediators that orchestrate the recruitment of large numbers of polymorphonuclear leukocytes (PMNs) from the pulmonary vasculature into the alveolar space (Zhang *et al.*, 2000). These recruited PMNs provide additional phagocytic capacities to support the alveolar macrophage and can also produce proinflammatory cytokines to help maintain high levels of inducible antimicrobial agents. Both these functions are crucial for the effective eradication of offending pathogens. The alveolar macrophage-derived substances capable of eliciting PMN migration into the airways include complement, chemokines such as interleukin-8 (IL-8) and arachidonic acid metabolites such as leukotriene B_4 (Zhang *et al.*, 2000). Under normal circumstances the induction of antimicrobials and the generation of an inflammatory response should clear an infection relatively quickly, before the host tissues suffer any major damage. The chronic inflammatory response associated with CF airway disease may be caused by deficits in one or more of these inducible defence mechanisms. These deficits may or may not be linked directly to mutated CFTR.

1.2 NITRIC OXIDE

Nitric oxide (NO) is a highly reactive gaseous mediator involved in many physiological processes. It was first described in the body as a pharmacological effector of endothelium-derived relaxation, through the activation of guanylate cyclase in vascular smooth muscle (Muhl *et al.*, 1995). NO is now recognized as a mediator of host defense, immune regulation, neurotransmission and inflammation (Moncada *et al.*, 1993). It is derived from the amino acid L-arginine by a family of enzymes known as the nitric oxide synthases (NOS). The oxidation of a terminal nitrogen of L-arginine by these enzymes produces citrulline and NO (Fig. 4). There are three isoforms of NOS:

endothelial (eNOS), neuronal (nNOS) and inducible (iNOS), which are products of different genes and show different localization, regulation, catalytic properties and inhibitor sensitivity. eNOS and nNOS are constitutively expressed and are collectively termed constitutive NOS (cNOS), whereas in most tissues iNOS is expressed only after induction by proinflammatory cytokines, microbes and/or bacterial products (Knowles & Moncada, 1994). All forms of NOS require reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavine dinucleotide (FAD), flavine mononucleotide (FMN) and tetrahydrobiopterin (BH_4) as cofactors. In addition, cNOSs are calcium and calmodulin-dependent (Green *et al.*, 1993). Regulation of iNOS activity shows no dependency on calcium.

Figure 4 The overall reaction catalysed by the nitric oxide synthase family (Alderton *et al.*, 2001)



Electrons (e^-) are donated by NADPH to the reductase domain of the enzyme and proceed via FAD and FMN redox carriers to the oxygenase domain. There they interact with the haem iron and BH_4 at the active site to catalyse the reaction of oxygen with L-arginine, generating citrulline and NO as products. Electron flow through the reductase domain requires the presence of bound calcium/calmodulin.

Constitutive NOS produce small amounts of NO (nM range) and are involved in homeostatic processes (Moncada *et al.*, 1991). Inducible NOS is responsible for the main production of NO in tissues (μM amounts) and is regulated at the transcriptional level (Morris & Billiar, 1994). Activation of cNOS can also be distinguished from that of iNOS on the basis of the length of time required to observe a significant increase in the levels of NO released and the length of time these levels can be maintained. cNOS requires no induction and increased NO is observed within seconds of a significant increase in intracellular Ca^{2+} accumulation. This increase is transient and fully inhibited by calmodulin antagonists (Stuehr & Griffith, 1992). Generation of NO by iNOS is highly regulated by the action of proinflammatory cytokines (Moncada & Higgs, 1993). The induction of iNOS can be measured at the RNA level within 30 minutes to 2 hours and increased NO release can be measured as soon as 3-4 hours post induction (Stefano *et al.*, 1998; Radomski *et al.*, 1990). Maximal production of NO by iNOS is commonly observed 6-24 hours post induction and increased NO release can be observed for 24-72 hours depending on cell type and species studied (Stefano & Magazine, 2001). iNOS is sensitive to inhibitors of DNA transcription and protein synthesis, such as actinomycin D and cycloheximide. Glucocorticoids (Radomski *et al.*, 1990; Pfeilschifter, 1991) and cytokines such as TGFβ, PDGF, IL-4 and IL-10 (Oswald *et al.*, 1992; McCall *et al.*, 1992; Ding *et al.*, 1990; Cunha *et al.*, 1992) have been found to inhibit iNOS expression, without affecting cNOS. The differences in the activities and regulation of the various forms of NOS suggest that the level of NO production can be regulated rapidly or slowly, depending on the needs of the organism.

NO produced by cNOS is associated with numerous physiological effects. In mammals, the activation of eNOS is linked to blood vessel relaxation, which helps to maintain

normal blood pressure (Welch *et al.*, 1995) and vascular integrity (Cosentino & Luscher, 1995). NO produced by nNOS acts as a non-conventional neurotransmitter and is an important regulator of cerebral vascular tone (Brian *et al.*, 1996). Induced NO can have a variety of effects, which may be beneficial or detrimental, depending on the amount produced, duration of activity and site of synthesis. It is believed to play a role in various pulmonary physiological processes, such as bronchodilation (Jorens *et al.*, 1993,) as well as being responsible for mediation of macrophage cytotoxicity and a part of the host response to sepsis and inflammation. A high level of NO production has also been associated with tissue damage, septic shock and airway inflammation of asthma (Lyons, 1995; Titheradge, 1999; Guo *et al.*, 1995). Increased iNOS expression and NO production have been demonstrated at sites of infection in animal models such as toxoplasmosis and leishmaniasis (Stenger *et al.*, 1996) and in human infections such as tuberculosis (Nicholson *et al.*, 1996). It seems that the induction of NO in many animal models is directly associated with the ability of the host to contain microbial proliferation, as abrogation of iNOS activity produces dramatic increases in microbial burden (Stenger *et al.*, 1996; MacMicking *et al.*, 1997). In support of this, *in vitro* studies of phagocytic cells and a variety of microbial targets have demonstrated cytokine-inducible microbiostatic or microbicidal activity which is L-arginine-dependent and inhibitible by competitive NO synthase inhibitors such as N^G-monomethyl-L-arginine (Adams *et al.*, 1990).

Induced NO can combine with reactive oxygen intermediates to generate a variety of antimicrobial molecular species, such as peroxynitrite ($OONO^-$), S-nitrosothiols (RSNO), nitrogen dioxide ($NO_2^\bullet$), dinitrogen trioxide (N_2O_3), dinitrogen tetroxide (N_2O_4), and dinitrosyl-iron complexes (DNIC) (Fang, 1997). Such interactions between reactive oxygen and nitrogen intermediates provide a molecular basis for synergy between the

respiratory burst and synthesis of NO. Interestingly, reaction products such as peroxynitrite, formed from the rapid interaction of NO and superoxide ($O_2^{\bullet-}$), can have greater cytotoxic potential than NO or $O_2^{\bullet-}$ alone. It is likely that different NO species have different affects on target microbial pathogens. For example NO itself does not possess antimicrobial activity for *S. typhimurium* or *E. coli* but S-nitrosothiols are bacteriostatic and peroxynitrite bactericidal for these organisms (Pacelli *et al.*, 1995; DeGroote *et al.*, 1995). In contrast, S-nitrosothiols and NO are microbicidal for *S. aureus* (Kaplan *et al.*, 1996), *L. major* (Assreuy *et al.*, 1994) and *G. lamblia* (Fernandes *et al.*, 1997), under conditions where peroxynitrite does not exert any antimicrobial effect.

DNA appears to be an important target of reactive nitrogen intermediates. $NO_2^{\bullet}$ and peroxynitrite can oxidatively damage DNA, resulting in abasic sites, strand breaks and a variety of other DNA alterations (Juedes *et al.*, 1996). There is also evidence that reactive nitrogen intermediates may interact with DNA repair systems and directly modify deoxyribonucleotides (Fang, 1997). These observations may explain the anti-proliferative effects of various NO species. NO and its intermediates have also been shown to inhibit key microbial enzymes by nitrosylation of reactive groups, such as iron-sulphur centres (Fe-S) and thiol groups (-SH), which are essential for enzyme catalytic function (Molina Y Vedia *et al.*, 1992; Cattel & Cook, 1993). This may account for the cytotoxic and cytostatic effects of NO on microorganisms. The cytotoxic activity of NO may play a role in tissue injury during inflammatory diseases and the generation of toxic radicals during its reaction with superoxide could contribute to this considerably. In particular, peroxynitrite has been shown to impair mitochondrial respiration and activate the poly-ADP ribose synthase (PARS) enzyme. This results in reduced NAD

and a decrease in the rate of glycolysis, electron transport and ATP generation, which ultimately leads to cell necrosis or apoptosis (Szabo *et al.*, 1997). To help to counteract these affects the body can protect against NO-mediated tissue damage by producing corticosteroids, growth factors and cytokines that are potent inhibitors of nitric oxide synthase expression (Laskin *et al.*, 1994; Liew, 1995).

1.2.1 Nitric Oxide and Airway Epithelium

NO has been detected in the exhaled air of normal patient volunteers and is increased in several inflammatory lung disorders, including asthma and bronchiectasis (Kharitonov *et al.*, 1994; Lundberg *et al.*, 1996). The various effects of NO on smooth muscle, neutrophils, lymphocytes, platelets and epithelial cells suggest that it may be a mediator of major importance in human airways, particularly in association with inflammatory airway disease (Barnes & Liew, 1995). In the context of infection it has been established that tissue macrophages from rodents use NO, along with other reactive nitrogen intermediates, to combat disease-causing agents such as *Mycobacterium tuberculosis* (MacMicking *et al.*, 1997) and *Leishmania major* (Wei *et al.*, 1995). These findings suggest that alveolar macrophages could be a potential source of exhaled NO in the human airway. However, iNOS has been very difficult to demonstrate in human macrophages derived from normal donors monocytes (Nathan, 1997). Conversely, in macrophages from patients with a wide variety of inflammatory diseases, iNOS has been more readily detected or induced (MacMicking *et al.*, 1997). It would seem that the macrophage priming environments that arise in infected or inflamed human hosts are difficult to recreate *in vitro* and further work is required to determine what contribution iNOS makes in human alveolar macrophages when it is fully expressed.

It is well established that airway epithelial cells are one of the major cell types involved in the defence of the lung against invading bacteria and other pathogens (Quie, 1986; Diamond *et al.*, 1996; Russell *et al.*, 1996) and several studies have demonstrated that iNOS is basally expressed at low levels in airway epithelial cells from rats and humans (Felley-Bosco *et al.*, 1994; Guo *et al.*, 1995; Rosbe *et al.*, 1996). The proinflammatory cytokines TNFα, IL-1β and IFNγ have been shown to increase significantly the level of iNOS expression in human primary epithelial cell cultures and in A549 cells, a type II alveolar carcinoma cell line (Asano *et al.*, 1994; Robbins *et al.*, 1994). Levels of nitrite, the stable end product of NO breakdown, were maximal 24 hours after cytokine stimulation and this increase in nitrite was consistent with an observed increase in iNOS protein and mRNA. These results suggest that increased airway epithelial cell expression of iNOS may well contribute to the increase in exhaled NO observed with inflammatory lung disease. The studies do not exclude the production of NO by activated human macrophages within the lung, but suggest that these cells may also play a role by releasing cytokines which stimulate NO release by lung epithelial cells. Consistent with this concept, alveolar macrophages are known to release increased amounts of TNFα and IL-1β in asthma (Gosset *et al.*, 1991; Borish *et al.*, 1992).

In support of the reports by Asano and Robbins in 1994, a study carried out on the SV-40 immortalized human bronchial epithelial (BEAS-2B) cell line demonstrated that cultures treated with TNFα, IFNγ and lipopolysaccharide (LPS) in combination, expressed iNOS mRNA which was associated with increases in supernatant nitrite concentration over 24 hours (Watkins *et al.*, 1997). However, this response diminished rapidly as the cells were successively passaged, with the response to cytokine stimulation becoming less predictable after only 4 passages. This latter finding is

consistent with work published by Guo and colleagues (1995) demonstrating that freshly isolated human bronchial epithelial cells lose their ability to synthesize iNOS mRNA within 72 hours. In a separate study on the bronchial epithelial cell line BET-1A, Guo and colleagues (1997) were unable to demonstrate any expression of iNOS in response to cytokines. These studies raise an interesting question as to the most suitable cell culture model for the study of NO biology in human airway epithelium.

1.2.2 Nitric Oxide and CF Airway Disease

It has been established that high NO levels correlate with chronically inflamed airways (Kharitonov *et al.*, 1994; Lundberg *et al.*, 1996). CF airway disease is associated with long lasting bacterial infections alongside chronic inflammation and it might be expected that CF lungs should also show elevated levels of NO. However, studies examining NO production in CF patients have proved inconclusive. Two studies demonstrate that exhaled NO levels are actually reduced in CF patients compared with normal subjects and patients with bronchiectasis or emphysema (Light *et al.*, 1989; Grasemann *et al.*, 1997). This is despite evidence that increased levels of the proinflammatory cytokines TNFα, IL-1β and IFNγ are present in the sputum from CF lungs (Bonfield *et al.*, 1995). In contrast, two different studies have suggested that there is increased nitrite in CF sputum (Linnane *et al.*, 1998; Francoeur & Dennis, 1995) and a further two report no differences in exhaled NO between CF patients and controls (Lundberg *et al.*, 1996; Balfour-Lynn *et al.*, 1996). Recently, it has been shown that iNOS expression is reduced in airway epithelium from CF mice (Kelley & Drumm, 1998) and the human CF bronchial epithelial cell line CFBE41o$^-$ (Meng *et al.*, 1998). These latest data lend support to the studies suggesting decreased levels of NO in CF airways.

The resulting effects of reduced levels of iNOS in the airway epithelium could underlie several aspects of the lung dysfunction which characterises CF airway disease. One of the most important is a compromised epithelial defence system. Epithelial cells appear to play a major role in the prevention of infection in the lung via generation of NO and its reactive nitrogen intermediates. Therefore, a lack of NO production could help to explain the persistence of microbial infections in CF. Smith *et al.* (1999), have demonstrated that NO can potentiate the toxicity of hydrogen peroxide, a major neutrophil-derived reactive oxygen species. If CF lungs do exhibit decreased levels of NO this could explain why some pathogens, particularly *Burkholderia cepacia* survive despite a vigorous neutrophil dominated inflammatory response.

The reasons behind the lack of iNOS expression in CF bronchial epithelium are unclear, but it is most likely a defect in signalling events leading to transcription. This could be at the level of cytokine receptors, their intracellular signalling pathways or transcription factors responsible for iNOS promotion (Meng *et al.*, 1997). It is unlikely to be a genetic defect as other cells in the CF lung are capable of expressing iNOS, such as alveolar macrophages and submucosal inflammatory cells (Meng *et al.*, 1997).

In addition to diminished antibacterial defences, a loss of iNOS expression in airway epithelial cells has also been linked to altered transepithelial transport of both sodium and chloride ions (Elmer *et al.*, 1999; Kamosinska *et al.*, 1997). Alongside chronic bacterial infection, sodium hyperabsorption is a common CF-associated airway abnormality (Kelley & Drumm, 1998). It is thought that in the healthy lung NO may act as a signalling agent to down-regulate sodium absorption via the amiloride sensitive epithelial sodium channel (ENaC) (Elmer *et al.*, 1999). NO is known to act through the activation of guanylate cyclases, thus stimulating the production of cGMP (Moncada *et*

al., 1991). The ability of cGMP to down-regulate amiloride sensitive sodium absorption has been demonstrated in several systems (Light *et al.*, 1989; Rozenweig & Seidman, 1991). Using murine airway epithelial cells Kelley and Drumm (1998) have demonstrated increased sodium absorption in response to the NOS inhibitor aminoguanidine. This study suggests that the loss of NO production in CF airways results in the subsequent loss of a cGMP signalling cascade and represents a potential mechanism for initiating the process of sodium hyperabsorption.

Since NO production has been shown to influence effectively both transepithelial chloride and sodium transport, it is plausible that CFTR conveys some regulation to iNOS production to further regulate basal ion transport properties. Using an *in vivo* mouse model Steagall *et al.* (2000), have demonstrated that iNOS expression is influenced at some level by the presence of active CFTR. Their study used *cftr* -/- mice where human CFTR had been introduced into the intestinal epithelium, but not the nasal epithelium. iNOS specific immunostaining of excised mouse tissue revealed strong iNOS expression in the epithelial cells of the ileum, but reduced staining in the nasal epithelium. This suggests a CFTR-related influence in the regulation of iNOS expression in epithelial cells. It is possible that in the absence of functional CFTR a change in cell signalling events occurs which alters the expression of iNOS in airway epithelium. Determining the mechanism of how a loss in CFTR function leads to reduced iNOS expression will hopefully lead to more effective therapeutic interventions.

1.3 CHEMOKINES

The term chemokine describes a family of closely related chemotactic cytokines, which are involved in attracting effector cells to inflammatory sites and also participate in the

activation of specific leukocytes (Lindley *et al.*, 1993). A wide range of cell types have been shown to produce chemokines, including lymphocytes, neutrophils, fibroblasts, endothelial cells and epithelial cells (Baggiolini *et al.*, 1994). They are released in response to appropriate stimulation, for example by bacterial LPS and the cytokines TNFα, IFNγ, and IL-1β. Members of the chemokine family are small, with molecular weights in the range of 8-12 kDa. They are structurally related in that they possess highly conserved cysteine residues that form disulphide bonds and establish the tertiary structure of the protein. Chemokines are divided into four subfamilies based on the arrangement of these conserved cysteine residues in the molecule (Oppenheim *et al.*, 1991).

The CXC subfamily has the first two NH_2-terminal cysteines separated by one nonconserved amino acid residue. In contrast, members of the CC subfamily exhibit two adjacent cysteine amino acid residues. The C subfamily has one lone NH_2-terminal cysteine residue, while the CX_3C subfamily has these cysteines separated by three intervening amino acids. There may be as many as forty to fifty human chemokines and most fall into the CXC and CC groups (Rollins, 1997). IL-8 is a prominent member of the CXC subfamily of chemokines whereas two important CC family members are monocyte chemoattractant protein-1 (MCP-1) and regulated on activation, normal T cell expressed and secreted (RANTES). The chemokine subfamilies exhibit cell selectivity with respect to chemoattraction. Members of the CXC subfamily primarily target neutrophils, whereas various members of the CC subfamily target monocytes, eosinophils and basophils. In particular IL-8 mediates neutrophil chemotaxis (Baggiolini *et al.*, 1989), MCP-1 mediates monocyte and basophil chemotaxis and activation (Leonard & Yoshimura, 1990), while RANTES induces chemotaxis of eosinophils, monocytes and CD45 RO^+ memory T lymphocytes (Schall *et al.*, 1990).

Chemokines induce cell migration and activation by binding to specific G-protein-coupled cell surface receptors on target cells (Premack & Schall, 1996; Murphy, 1994). The ligation of these receptors activates multiple intracellular signalling pathways that regulate the intracellular machinery necessary to propel the cell in its chosen direction. To date at least six human CXC chemokine receptors (CXCR1 through to CXCR6) and eleven human CC chemokine receptors (CCR1 to CCR11) have been described (reviewed by Watson, 2002). CXC chemokine receptors are found predominantly on neutrophils, hence the selectivity of CXC chemokines for this cell type. In contrast, CC chemokine receptors are expressed on a much wider range of cells, including lymphocytes, monocytes, macrophages, eosinophils and basophils (Wells *et al.*, 1998).

The migration of leukocytes from blood vessels to an inflammatory site is a highly regulated multi-step process, involving a series of coordinated interactions between leukocytes and endothelial cells. Four sequential steps can essentially define the process: rolling, triggering, adhesion and migration. Chemokines have been shown to participate at several stages in this process (Adams & Shaw, 1994). Initially, the expression of selectins by both endothelium and leukocytes facilitates the movement of leukocytes along the surface of endothelial cells. Chemokines trigger these leukocytes to express integrins on their cell surface. The integrins interact with adhesion molecules, which are expressed on the surface of activated endothelial cells. This interaction promotes strong adhesion of the leukocytes to the vessel wall. Finally, the leukocytes migrate into the underlying tissue along a chemotactic gradient. Different subsets of leukocytes express particular integrins on their cell surface, which interact with specific adhesion molecules. This results in the selective recruitment of leukocytes into inflamed tissue.

The increased secretion of chemokines has been detected in a wide variety of inflammatory diseases, such as acute respiratory distress syndrome (Chollet-Martin *et al.*, 1993), asthma (Bousquet *et al.*, 1990) and Crohn's disease (Garcia-Zepeda *et al.*, 1996; Reinecker *et al.*, 1995; Grimm & Doe 1996). The type of inflammatory infiltrate that characterises each particular disease is controlled, in part, by the subgroup of chemokines expressed in the diseased tissue. This suggests that various chemokines and their receptors might provide novel targets for future therapeutic intervention in inflammatory conditions.

1.3.1 Interleukin-8 and CF Airway Disease

The origin of airway inflammation in CF has been the subject of much debate following the finding of neutrophil dominated inflammation in the absence of bacterial infection in the bronchoalveolar lavage (BAL) fluid of CF infants (Wilmott *et al.*, 1990; Ramsey *et al.*, 1991; Birrer *et al.*, 1994; Balough *et al.*, 1995; Khan *et al.*, 1995). These observations lead to the proposal that an inability to downregulate inflammation is intrinsic in CF (Cantin, 1995). Several pieces of data support this concept. Inhibition of inflammation by high dose ibuprofen or steroids is actually beneficial to patients with CF (Eigen *et al.*, 1995; Konstan *et al.*, 1995). Secondly, increased IL-8 has been found in the airways of young children with CF compared with the airways of other young children without CF but with a comparable airway burden of bacteria (Muhlebach *et al.*, 1999). Finally CF mice die earlier from chronic *Pseudomonas* infection than their non-CF littermates. This increased death rate is accompanied by increased proinflammatory cytokines in BAL fluid, but not by an increased bacterial burden (Van Heeckeren *et al.*, 1997).

However, Armstrong and coworkers (1997), argue against this proposal. They carried out a longitudinal BAL study of infants with CF and found that in the absence of infection, the CF children had no increase in neutrophils or IL-8 compared with controls. This suggests that at a very early age, little excess IL-8 or inflammation is present in children with CF without an infectious stimulus. The authors suggested that the methods used in previous studies might have been insufficiently sensitive to detect infection. In particular, the usual practice of sampling only one or two segments of the lung during BAL was thought to be inadequate. Dakin *et al.* (2002), sampled three lung lobes when collecting BAL fluid, in order to maximize the potential of detection of infection and inflammation. Their findings did not exclude the possibility of a degree of intrinsic inflammation in CF, but did describe a very significant relationship between infection and inflammation, with the level of pathogens cultured from the BAL fluid explaining most of the variation in inflammatory cells and a third of the variation in IL-8 levels.

One potential link between the tendency towards infection and excessive inflammation is the response of airway epithelial cells to contact with *Pseudomonas aeruginosa*, an organism that shows increased binding to CF cells in culture compared with non-CF cells (Imundo *et al.*, 1995). This response may arise from the interaction of pilin on the surface of *Pseudomonas* with asialo-GM_1 receptors, which are increased on the surface of CF airway epithelial cells (Saiman & Prince, 1993). The main product from *Pseudomonas* responsible for increasing IL-8 expression in airway epithelial cells has been identified and was initially described as a low molecular weight, heat stable, non-protein, non-LPS molecule (Massion *et al.*, 1994). More recently this molecule was further characterised as N-3-oxododecanoyl homoserine lactone (3-O-C12-HSL), a *Pseudomonas aeruginosa* autoinducer (Smith *et al.*, 2001). *Pseudomonas*

autoinducers are known to induce the transcription of genes that lead to the production of bacterial virulence factors (Van Delden & Iglewski, 1998). The effects of these virulence factors are known to contribute directly to the persistence and proliferation of the bacteria in the airway, which leads to induction of intense inflammation during infection. There is also evidence that when *Pseudomonas* is grown as a biofilm, the ratio of autoinducers produced is different to that of planktonic bacteria and the concentration of 3-O-C12-HSL is significantly higher (Smith *et al.*, 2001). Biofilms are initiated by the attachment of individual planktonic bacteria to a membranous surface, such as the lung epithelium. As a result of cell-cell interactions, a matrix of hydrated polymers is produced and this eventually develops into an elaborate three-dimensional structure (Costerton *et al.*, 1995; Watnick & Kolter, 2000). Bacteria growing as a biofilm are able to withstand host immune responses and exhibit increased resistance to antibiotics and biocides when compared with the same cells growing in liquid culture (Xu *et al.*, 2000). Recent work has demonstrated that *Pseudomonas aeruginosa* exists as a biofilm in the CF lung (Singh *et al.*, 2000). The concentration of autoinducers, such as N-3-oxododecanoyl homoserine lactone, may therefore by very high in *Pseudomonas* infected CF airways. If this were the case, it would have the effect of exacerbating any inflammatory response.

Using both matched and corrected airway epithelial cell lines, it has been consistently demonstrated that expression of IL-8 after *Pseudomonas* stimulation is increased in cells with CFTR dysfunction (DiMango *et al.*, 1995; Bryan *et al.*, 1998; Tabary *et al.*, 1999). A recent study has shown that this increase in IL-8 production is attenuated when the cells are incubated with a mutant strain of *Pseudomonas* that lacks pili and therefore cannot bind to the asialo-GM_1 receptors (Kube *et al.*, 2001). However, even with the mutant strain the CF-phenotype cells still showed a significant increase over

control cells, suggesting that factors other than *Pseudomonas* binding to the epithelial cell affect production of IL-8. The authors also demonstrated that the time course response differed between the normal and CF-phenotype cells used in this study, with the latter continuing increased IL-8 production long after it had ceased or slowed in the normal cells. This suggests that the mechanisms limiting IL-8 production in normal cells may be absent or altered in CF.

Studies examining the effects of proinflammatory cytokines on IL-8 production by CF and normal airway epithelial cells have produced results that contradict those from the bacterial experiments. Black *et al.* (1998), found that primary cultures of nasal epithelial cells from young children released increased amounts of IL-8 soon after a short exposure to TNFα. However, comparison of cultures from children with CF and similar cultures from children without CF showed no differences in the magnitude or duration of this IL-8 response. A further finding was that immortalised CF cells did not show increased IL-8 responses compared with retrovirally corrected cells derived from the same line. In fact the trend was actually towards lower IL-8 production in the cell lines with CFTR dysfunction. Other studies using a range of CF and non-CF primary and immortalised airway epithelial cell lines stimulated with a range of proinflammatory cytokines have found similar results (Schwiebert *et al.*, 1999: Massengale *et al.*, 1999). In contrast two studies have reported increased IL-8 expression by CF airway epithelial cells in the presence and absence of proinflammatory cytokines (Ruef *et al.*, 1993; Stecenko *et al.*, 1997). However, these authors examined only two epithelial cell lines, did not compare IL-8 expression between CF and non-CF primary airway epithelial cells and did not demonstrate that the corrected cell lines they used exhibited wildtype CFTR activity. A more recent study by Venkatakrishnan *et al.* (2000), demonstrated little IL-8 production by CF airway epithelial cells in the absence of an inflammatory stimulus, but

an augmented response compared to normal cells after TNFα stimulation. The authors suggest that differences in the duration of stimulation by TNFα may account for the differences between their findings and those of Black and colleagues (1998). In the Venkatakrishnan study, TNFα was continuously present in the cell culture medium, whereas in the study by Black *et* al. (1998), cell stimulation involved exposing the cells for one hour to cytokines.

Taken together the bacterial and cytokine studies suggest that different induction pathways may lead to IL-8 production depending upon the nature of the stimulus. It also appears that enhanced IL-8 expression by CF airway epithelial cells may not be the source of the increased IL-8 levels detected in CF patient BAL fluid. It has been demonstrated that large numbers of macrophages are present in the BAL fluid of infected CF patients (Khan *et al.*, 1995). These macrophages could be the primary source of IL-8 in CF airways. Additional studies are needed to further investigate IL-8 secretion in other airway epithelial cell lines and primary tissue and to determine directly whether defective CFTR production and surface expression alter the secretion of IL-8.

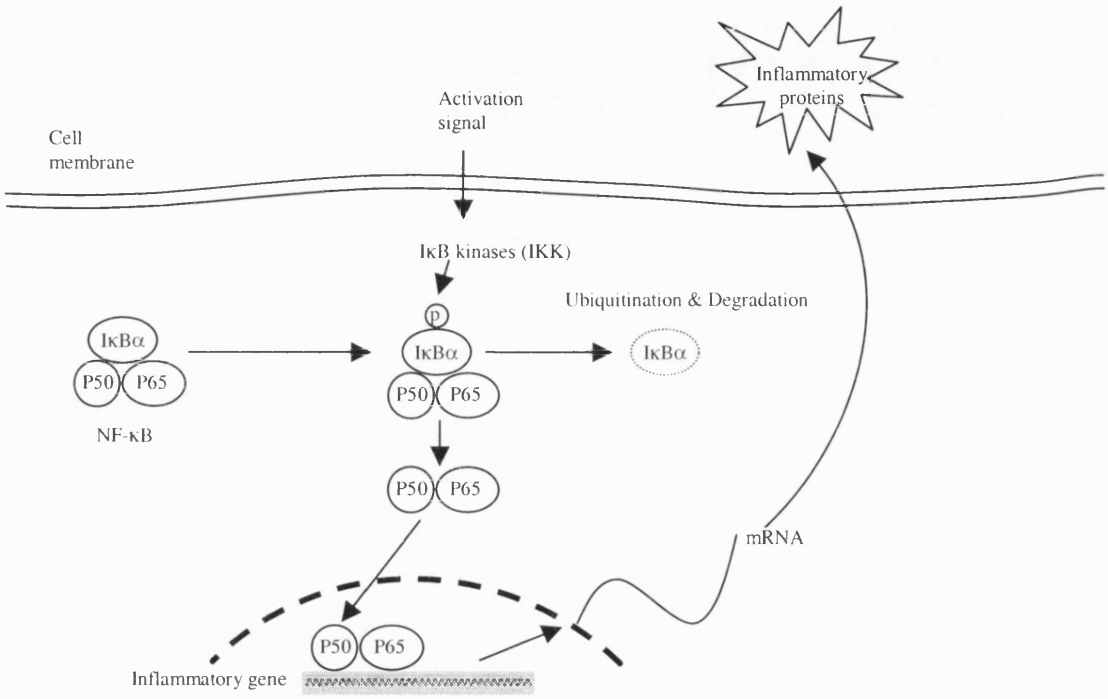
1.4 EPITHELIAL TRANSCRIPTION FACTORS AND INFLAMMATION

The binding of proinflammatory cytokines or microbial products to the surface of epithelial cells leads to the activation of transcription factors within the cells. This in turn leads to the transcription of genes involved in inflammation, cell growth control and apoptosis. Nuclear factor-kappaB (NF-κB) is one of the main transcription factors which regulates the inducible expression of genes encoding proteins involved in the

modulation of inflammatory host defense processes in eucaryotic cells (Barnes & Karin, 1997).

NF-κB was first identified as a regulator of the expression of the kappa light-chain gene in murine B-lymphocytes (Sen & Baltimore, 1986), but has since been found in many different cell types. In its active form it is a heterodimer usually consisting of a p50 subunit and a p65 (relA) subunit. Other subunits, such as rel, relB, v-rel and p52 may also be part of activated NF-κB and it is likely that the different forms of NF-κB may activate different sets of target genes (Siebenlist *et al.*, 1994; Baeuerle & Baltimore, 1996). In unstimulated cells NF-κB is sequestered in the cytoplasm through interaction with inhibitory kappaB (IκB) proteins of which there are several forms, IκBα, IκBβ, IkBε and Bcl-3 (May & Ghosh, 1997). This association with an inhibitory protein prevents NF-κB from entering the nucleus. Upon cell stimulation a kinase complex known as IκB kinase (IKK), phosphorylates IκB on specific serine residues, causing its subsequent ubiquitination and rapid degradation by proteosomes (DiDonato *et al*, 1996; Chen *et al*, 1996). Once NF-κB has been liberated, the complex translocates into the nucleus and transcriptionally activates target genes by binding to responsive elements in the DNA called κB motifs (Fig. 5).

Figure 5 The activation of NF-κB by IκB kinases. Most work has focused on the predominant form of NF-κB, the p65/p50 heterodimer, and its association with IκBα (adapted from Barnes & Karin, 1997).



It has been demonstrated that activated NF-κB can induce the synthesis of IκBα by binding to κB motifs within its promoter (Arenzana-Seisdedos *et al.*, 1995). Newly synthesised IκBα enters the nucleus, binds to active NF-κB and the whole complex returns to the cytoplasm thus terminating the activation of gene expression. In contrast the synthesis of IκBβ is not induced by NF-κB, which suggests that NF-κB is likely to be active for a more prolonged period in the types of cells where IκBβ predominates (Thompson *et al.*, 1995). Little is known about the regulation of the other IκB isoforms.

NF-κB can be activated within minutes by a variety of stimuli, including proinflammatory cytokines, growth factors and stress inducers (Baldwin, 2001). Several signal-transduction pathways may be involved, but they all ultimately act by phosphorylating and therefore degrading IκB. Interestingly, products of the genes that are regulated by NF-κB can also cause its activation. For example IL-1β and TNFα both activate and are activated by NF-κB (Barnes & Karin, 1997). This type of positive regulatory loop may amplify and perpetuate local inflammatory responses.

The activation of NF-κB leads to a coordinated increase in the expression of many genes whose products mediate inflammatory and immune responses. However it is not the only transcription factor involved in regulating these genes. It frequently functions together with other transcription factors such as activator protein-1 (AP-1). The AP-1 transcription factor is a complex composed of proteins of the *fos* and *jun* proto-oncogene families. These proteins need to dimerize to promote binding of the complex to AP-1 recognition sites in target genes (Hunter & Karin, 1992). NF-κB and AP-1 act on genes for proinflammatory cytokines, chemokines, enzymes that generate mediators of inflammation, immune receptors and adhesion molecules (Barnes & Karin, 1997). Thus, they play a key part in the initial recruitment of leukocytes to sites of inflammation and in the induction of enzymes such as iNOS.

Most of our current understanding of the transcriptional regulation of iNOS has been derived from analysis of the 5'-flanking region of murine (and to a considerably lesser extent, human) iNOS promoter. Although iNOS isoforms are highly conserved between species, suggesting that they are all products of the same gene, there is less homology in the 5'-flanking region (Xie *et al.*, 1992; Geller *et al.*, 1993; Chartrain *et al.*, 1994;

Nunokawa *et al.*, 1994). Moreover, iNOS gene transcription seems to be regulated differently between various human cell lines (Charles *et al.*, 1993; Geller *et al.*, 1993; Linn *et al.*, 1997). This probably explains why cytokine combinations leading to iNOS induction seem to vary between species and between cell types in the same species (Charles *et al.*, 1993; Geller *et al.*, 1993; Lowenstein *et al.*, 1993; Nunokawa *et al.*, 1994).

In the mouse two NF-κB binding sites have been identified in the 5'-flanking region of the iNOS promoter, with the more downstream site accounting for LPS induction of iNOS. An upstream site contains enhancer regions with binding sites for γ-activated site element and interferon regulatory factor (IRF)-1 response element that account for the potentiation of LPS-stimulated promoter induction by IFNγ (Lowenstein *et al.*, 1993). Cytokine regulation of human iNOS promoter includes a considerably larger region of 5'-flanking sequence compared with the mouse (DeVera *et al.*, 1996; Taylor *et al.*, 1998; Ganster *et al.*, 2001; Mellott *et al*, 2001). Computer analysis of the 8296bp 5' region of the human iNOS gene has identified multiple copies of IFNγ response elements, two copies of AP-1 and two copies of NF-κB response elements. It should be noted that while NF-κB does have a role in the induction of human iNOS it appears to be less important than its role in the regulation of the murine gene (Chu *et al.*, 1998). Recent studies have suggested that human iNOS regulation shows greater reliance on IRF-1 and the activation of the transcription factor signal transducers and activators of transcription (STAT)-1, both part of the IFNγ signalling pathway (Bories *et al.*, 1999; Guo *et al.*, 1997). However, these requirements may differ considerably between cell types and cell lines. The differences in the regulation of human and murine iNOS call into question the suitability of using animal models in nitric oxide research. It would

appear that data obtained from mouse studies do not accurately reflect signalling mechanisms in human cells.

IL-8 expression is regulated at both the transcriptional and the posttranscriptional levels. The former is primarily mediated by multiple elements in the 5' promoter region of the IL-8 gene, including two IRF-1 elements, an AP-1 sequence, an AP-2 site, an AP-3 site, a CCAAT/enhancer binding protein (C/EBP) sequence and an NF-κB-nuclear factor of interleukin-6 (NF-IL-6) overlapping sequence (Mukaida *et al.*, 1990; Kunsch & Rosen, 1993; Matsusaka *et al.*, 1993; Oliveira *et al.*, 1994). Activation of NF-κB is the most crucial step for IL-8 gene transcription in most cells, however the transcription factors with which it cooperates may vary in a cell type specific manner (Mukaida *et al.*, 1994). It also seems that the molecular mechanisms involved in IL-8 gene transcription differ, even in the same cell, depending on the employed stimuli. For example, it has been demonstrated that AP-2 and NF-κB are the most prominent activators of IL-8 when airway epithelial cells are stimulated with the *Pseudomonas* autoinducer 3-O-C12-HSL. However, when these same cells are stimulated with TNFα and IL-1β only NF-κB is necessary for maximal IL-8 induction (Smith *et al.*, 2001).

It has been suggested that the synergistic effect of different cytokines on iNOS and IL-8 induction results from physical interactions between different transcription factors. These interactions were thought to lead to the formation of a highly stable multiprotein complex with enhanced DNA binding affinity (Taylor *et al.*, 1998). However, a more recent study proposes that synergism may be secondary to structural alterations in DNA conformation that occur when transcription factors bind to their specific promoter

regions. These alterations may lead to bending or looping of the DNA and thus enhanced transcription (Saura *et al.*, 1999).

Not surprisingly, NF-κB has been found to be highly active in a wide range of inflammatory diseases, including airway diseases such as asthma (Hart *et al.*, 1998). Recently it has been reported that NF-κB is constitutively active in the CF airway epithelial cell line IB3 and in human CF bronchial gland (HBG) cells compared to normal cells (DiMango *et al.*, 1998; Tabary *et al.*, 1999). These data support the observations that cells with CFTR mutations are more readily stimulated to produce IL-8 (DiMango *et al.*, 1995; Bryan *et al.*, 1998; Tabary *et al.*, 1999). DiMango and colleagues (1998), suggest that the aberrant trafficking of mutant CFTR and its accumulation in the endoplasmic reticulum (ER) could overwhelm normal cell degradation pathways provoking stress activation of NF-κB. They hypothesise that this endogenous activation, together with increased bacterial adherence to CF cells (Imundo *et al.*, 1995), creates the inflammatory environment characteristic of CF airway disease. A more recent study (Weber *et al.,* 2001) lends support to this theory. Weber and colleagues demonstrated that stable transfection of Chinese hamster ovary (CHO) cells with ΔF508 CFTR resulted in a significant increase in NF-κB activation compared with CHO cells stably transfected with wild-type CFTR. They also reported NF-κB activation in airway epithelial cell lines that lacked CFTR chloride channel function, but did not accumulate mutant protein in the ER. This activation of NF-κB correlated with elevated IL-8 expression. The authors concluded that mutated CFTR could activate NF-κB by two independent mechanisms: cell stress associated with mistrafficking or effects directly due to lack of CFTR chloride channel function. However, there is an important caveat to these studies. Although it has been reported that ΔF508 CFTR accumulates

in the ER due to abnormal maturation and folding (Cheng *et al.*, 1990; Welsh & Smith, 1993), other researchers have demonstrated ΔF508 CFTR expression levels in airway and intestinal epithelium that are comparable to wild-type (Kalin *et al.*, 1999). This may impact on the relevance of the ER overload hypothesis in airway epithelial cells. In contrast to the studies outlined above, Venkatakrishnan *et al.* (2000), found little NF-κB activation in unstimulated CF airway epithelial cells compared to corrected control cell lines. However, they did demonstrate increased NF-κB activation in the CF cell lines compared to control after TNFα stimulation. They refute the ER overload hypothesis and instead suggest that these alterations in NF-κB activity are associated with changes in IκBβ regulation. To date studies examining the relationship between mutant CFTR and NF-κB have only been carried out on a limited number of cell lines, so it remains to be determined whether these findings are applicable to other cell lines and primary tissue from CF patients.

The interactions between the various transcription factors involved in the induction of inflammatory genes are well on the way to being understood and blocking the activation of proteins such as NF-κB may help in certain inflammatory disorders. However, NF-κB does provide a useful role in cellular homeostasis and there could be serious side effects in blocking its activation in the long term. The intermediate events between cytokines binding to membrane receptors and the activation of transcription factors are incompletely defined and may involve numerous signalling pathways. Elucidation of these pathways will hopefully enable an approach to regulating inflammation, which aims to restore a balance of inflammatory mediators, rather than completely inhibiting their action.

1.5 EPITHELIAL CELL SIGNALLING AND CF

Signal transduction involves the coupling of ligand-receptor interactions to intracellular events. These events include phosphorylation cascades that are regulated by tyrosine kinases, serine/threonine kinases and/or lipid kinases. The protein phosphorylations change enzyme activities and protein conformations, which in turn alter cellular activity and change the program of gene expression within a cell. It is possible that these pathways are intrinsically altered in CF airway epithelia.

1.5.1 The Role of Mitogen-Activated Protein Kinases in NF-κB Regulation

Among the signalling cascades involved in the response of cells to growth factors, cytokines or environmental stress are members of the mitogen-activated protein kinase (MAPK) family. Mitogen-activated protein kinases (MAPKs) are signalling proteins that are regulated by tyrosine and threonine phosphorylation mediated by distinct upstream MAPK kinases (MEKs). MEKs are themselves phosphorylated and activated by specific MEK kinases (Robinson & Cobb, 1997). These sequences of phosphorylation allow the amplification of external signals with each phosphorylating step. In mammalian cells, at least three different subfamilies of MAPKs have been described. They include the p42/p44 or extracellular signal-regulated kinases (ERK1/2), which are activated by growth factors and c-jun amino-terminal kinase (JNK) and p38, which are activated by cytokines and cellular stress. Each MAPK class responds to distinct stimuli and induces specific biological responses. This specificity is maintained throughout each cascade by selective enzyme-substrate interactions (Marshall, 1994). The activated MAPKs may then regulate the activity of transcription factors to control gene expression.

To date only a limited amount of work has been carried out on the role of MAPKs in airway epithelial cell inflammatory responses. However, it has been shown in other systems that activation of the ERK1/2 MAPK pathway results in the phosphorylation of IκBα and thus the activation of NF-κB (Blenis & Resh, 1993; Ghoda *et al.*, 1997). Smith and colleagues (2001), suggest that the activation of NF-κB and the production of IL-8 found in airway epithelial cells stimulated with the *Pseudomonas* autoinducer 3-O-C12-HSL occur through a similar mechanism. They demonstrated that 3-O-C12-HSL was able to up-regulate both ERK1 and 2 in the cytoplasm of 16HBEo$^-$ cells. When an inhibitor specific for the upstream kinase of ERK1/2 was added to the cultures, 3-O-C12-HSL stimulation of both IL-8 and NF-κB were inhibited. The authors did not show any involvement of JNK or p38 MAPK pathways in NF-κB activation in this cell line. Consistent with these results, Ratner *et al.* (2001), observed that ERK1/2 inhibition caused a significant reduction in NF-κB activation and IL-8 production in 1HAEo$^-$ airway epithelial cells in response to stimulation with *Pseudomonas aeruginosa.* However, in contrast with Smith *et al.* (2001), they also demonstrated a role for the p38 MAPK pathway in this NF-κB activation and IL-8 induction.

Recently, Kristof *et al.* (2001), showed that the inhibition of p38 and ERK1/2 MAPKs caused a decrease in the activity of human iNOS promoter in A549 airway epithelial cells transiently transfected with luciferase reporter plasmids. Reduced cytokine and LPS/IFNγ-stimulated AP-1 binding accompanied this decrease in activity, whereas binding to the upstream NF-κB (relA/relA) sequence was increased. These data are consistent with an essential role for MAPKs in AP-1, but not NF-κB, -dependent human iNOS activation. The mechanism by which binding to the NF-κB upstream site was

augmented by the ERK1/2 and p38 inhibitors is not known, although one group have reported a reduction of NF-κB activity by activated MAPKs (Alpert *et al.*, 1999).

It is unclear as to why microbial activation of NF-κB and induction of IL-8 should involve MAPKs, whereas cytokine and LPS/IFNγ activation of NF-κB associated with iNOS induction do not. It is possible that these differences are the result of different NF-κB dimers binding to particular -κB motifs in the promoter regions of target genes in response to specific stimuli. However, more work into the involvement of MAPKs in NF-κB and AP-1 activation is needed if differences such as these are to be fully explained.

1.5.2 The Role of Phosphatidylinositol 3-kinase in NF-κB Activation

Phosphatidylinositol 3-kinases (PI3Ks) are a ubiquitously expressed enzyme family that participate in a wide range of cellular processes, including cell growth and transformation, differentiation, motility, insulin action and cell survival (Toker, 2000). They belong to a subfamily of lipid kinases and catalyse the addition of a phosphate molecule specifically to the 3'-OH position of the inositol ring of membrane-bound phosphoinositide (PI) lipids. The 3'-OH species found in mammalian cells include the single phosphorylated phosphatidylinositol-3-monophosphate (PtdIns-3-P); the double phosphorylated forms phosphatidylinositol-3,4-bisphosphate (PtdIns-3,4-P_2) and phosphatidylinositol-3,5-bisphosphate (PtdIns-3,5-P_2) and finally the triple phosphorylated form phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P_3). Inositol lipids phosphorylated at this position are termed D-3 lipids and they make up less than 0.25% of the total inositol-containing lipids within the cell. This is consistent with the

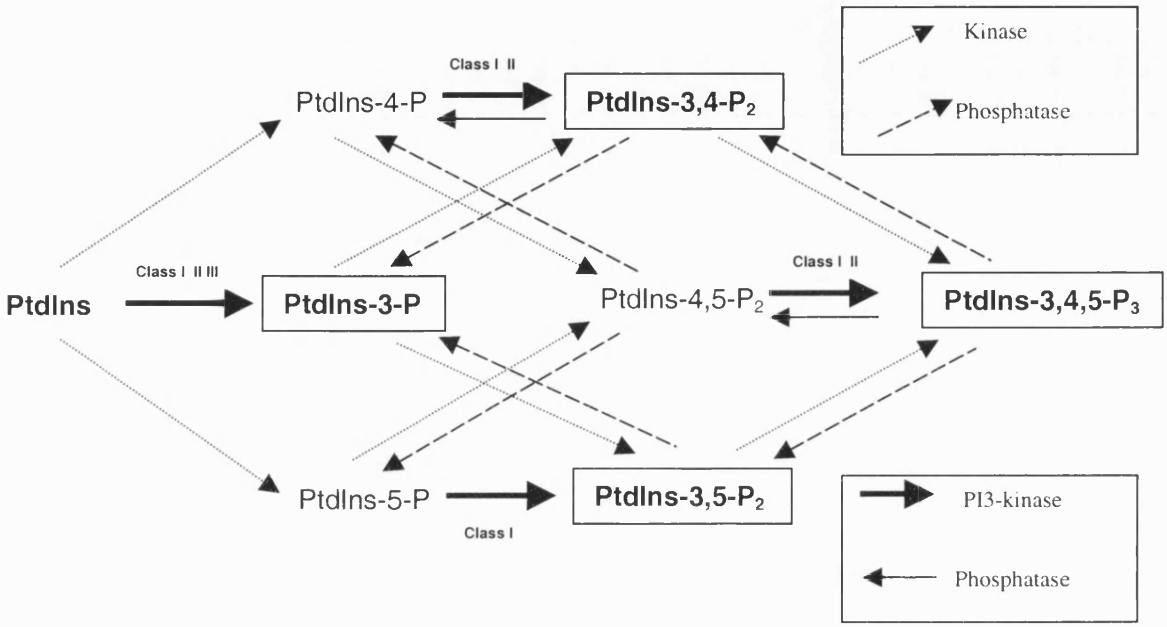
idea that these lipids have specific regulatory functions inside the cell as oppose to structural functions (Rameh & Cantley, 1999). Members of the PI3K family have been grouped into three classes according to their primary structure, their regulation and the molecules they utilise as substrates (reviewed in Vanhaesebroeck *et al.*, 1997).

Class I PI3Ks are heterodimers consisting of a 110 kDa catalytic subunit (p110) and an 85 kDa adaptor/regulatory subunit (p85). *In vitro*, this class of PI3K can utilise PtdIns, PtdIns-4-P and PtdIns-4,5-P_2. However, in cells their preferred substrate appears to be PtdIns-4,5-P_2 (Fig. 6). All class I PI3K members are also capable of binding to the monomeric G-protein Ras. The significance of this interaction in PI3K signalling is unclear, but it may be a way of tethering the enzyme to the membrane. Class I PI3Ks can be further subdivided into class I_A and class I_B enzymes. In class I_A PI3Ks, the p110 subunit is complexed to an adaptor protein that has two Src-homology-2 (SH2) domains. These SH2 domains bind to phosphorylated tyrosine residues that are generated by activated tyrosine kinases in receptors and various adaptor proteins. This is believed to allow translocation of cytosolic PI3Ks to the membrane where their lipid substrates and Ras reside. All mammalian cell types so far investigated express at least one class I_A PI3K isoform and stimulation of almost every receptor that induces tyrosine kinase activity also leads to class I_A PI3K activation (Stephens *et al.*, 1993; Fry, 1994; Wymann & Pirola, 1998). The only class I_B PI3K identified to date consists of a p110γ catalytic subunit complexed with a 101 kDa regulatory protein (p101). P110γ/p101 heterodimers are activated by the $\beta\gamma$ subunits of heterotrimeric G-proteins (Krugmann *et al.*, 1999) and have only been found in any abundance in mammalian white blood cells.

Class II PI3Ks are large molecules (> 170 kDa) that do not associate with an adaptor protein or bind to Ras. They are fairly ubiquitous and although little is known about their regulation, tyrosine kinase activity and heterotrimeric G-proteins may be involved (Arcaro *et al.*, 1998). *In vitro* class II PI3Ks can utilise PtdIns, PtdIns-4-P and PtdIns-4,5-P_2 as substrates, although the preference is for PtdIns and PtdIns-4-P (MacDougall *et al.*, 1995; Domin *et al.*, 1997) (Fig. 6). It is not clear which lipids class II PI3Ks make *in vivo.*

Class III PI3Ks are the homologues of the yeast vesicular protein-sorting protein Vps34p. These PI3Ks can only use PtdIns as a substrate *in vitro* (Fig. 6) and they are probably responsible for the generation of most of the PtdIns-3-P found in cells. The cellular levels of PtdIns-3-P remain fairly constant at all times, suggesting that the physiological processes in which class III PI3Ks are involved are not acutely triggered by cellular stimulation.

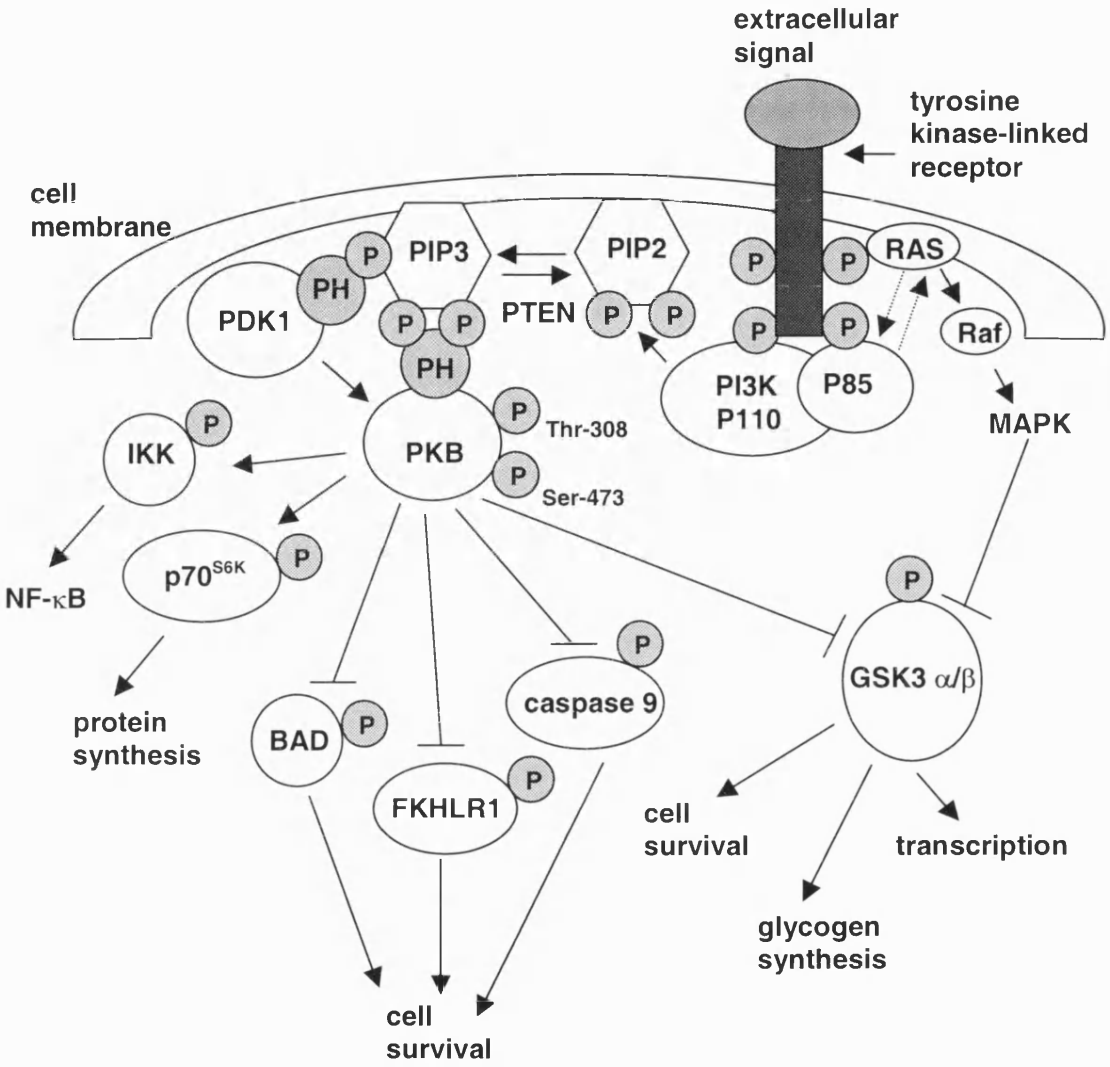
Figure 6 Pathways outlining the synthesis of phosphoinositide 3-kinase lipids (adapted from Rameh & Cantley, 1999)



Phosphoinositide 3-kinase lipids are generated in response to hormones, growth factors and other extracellular stimuli. They can lead to a wide range of cellular responses by interacting with effector proteins, which in turn modulate a large number of cellular targets (Corvera *et al.*, 1998). The main products of PI3K activity, PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3, are known to bind to proteins containing pleckstrin-homology (PH) domains (Vanhaesebroeck & Waterfield, 1999). The serine/threonine protein kinase B (PKB), also referred to as Akt, contains a PH domain and is the most well characterised target of PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 (Rameh & Cantley, 1999). In addition to binding the phosphoinositide lipids, full *in vivo* activation of PKB also depends upon the

phosphorylation of a threonine residue (Thr-308) in its kinase domain and a serine residue (Ser-473) in its C-terminal tail (Alessi *et al.*, 1996). Phosphorylation of these residues is known to be dependent on PI3K. The Thr-308 kinase, phosphoinositide-dependent kinase 1 (PDK1), has recently been identified and like PKB it contains a PH domain and binds with high affinity to PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 (Stephens *et al.*, 1998). It has been proposed that PI3K lipid products are involved in recruitment of PKB and its upstream kinases to the membrane, via their PH domains and also in promoting conformational changes in PKB that expose Thr-308 and Ser 473 to phosphorylation by PDK1 and other such kinases (Franke *et al.*, 1997; Downward, 1998). Once activated PKB leaves the plasma membrane to phosphorylate a variety of intracellular substrates. The phosphorylation of these substrates triggers a wide range of cellular activities (Fig. 7).

Figure 7 An overview of the major class I PI3K signalling pathways and functions



Many additional downstream targets of PI3K have been identified; those shown here have particularly well-defined roles in cell survival and proliferation.

Abbreviations: (P) phosphate; PIP3, PtdIns-3,4,5-P_3; PIP2, PtdIns-4,5-P_2; PI3K, phosphatidylinositol 3-kinase; PDK1, phosphoinositide dependent kinase 1; PKB, protein kinase B; PH, pleckstrin-homology domain; GSK3, glycogen synthase kinase-3; BAD, Bcl-2 antagonist of cell death; FKHLR1, forkhead transcription factor, $p70^{S6K}$, p70 S6 kinase; IKK, IκB kinase; NF-κB, nuclear factor-kappaB; MAPK, mitogen-activated protein kinase; PTEN, phosphatase and tensin homologue deleted on chromosome ten.

A great deal of evidence has emerged linking PI3K/PKB signalling to cell survival (reviewed by Coffer *et al.*, 1998). Studies using primary human leucocytes have established that survival factors such as IL-3 can suppress apoptosis by activating PKB. Once activated, PKB phosphorylates and inactivates key components of apoptotic pathways such as the proapoptotic Bcl-2 family member, BAD and the protease caspase 9 (Datta *et al.*, 1997; del Peso *et al.*, 1997; Cardone *et al.*, 1998). Recently it was demonstrated that activated PKB also inhibits apoptosis in a transcription-dependent manner in human B cells, by phosphorylating and inactivating the forkhead transcription factor family member, FKHRL1 (Brunet *et al.*, 1999). Within the nucleus, FKHRL1 probably triggers apoptosis by inducing the expression of genes that are critical for cell death, such as the Fas ligand gene. Phosphorylation by PKB leads to the translocation of FKHRL1 from the nucleus into the cytoplasm away from its transcriptional targets.

Activated PKB has also been shown to activate the transcription factor NF-κB in human leucocytes and a range of cancer cell lines (Ozes *et al.*, 1999; Romashkova & Makarov, 1999; Kane *et al.*, 1999; Madrid *et al.*, 2000). Although the induced nuclear translocation of NF-κB has been highly regarded as the principal method to activate NF-κB-dependent gene expression, an alternative mechanism of NF-κB activation is emerging that involves the phosphorylation of the p65 subunit. For example, it has been shown that the proinflammatory cytokines IL-1β and TNFα lead to the phosphorylation of p65 and the subsequent transactivation of NF-κB through pathways distinct from induced nuclear translocation (Bird *et al.*, 1997; Wang & Baldwin, 1998). In 1999, Sizemore and colleagues established that this transactivation of NF-κB occurs in a PI3K/PKB-dependent manner. More recently Madrid *et al.* (2001), suggested that

the ability of PKB to stimulate the transactivation potential of the p65 subunit of NF-κB in murine fibroblasts and human kidney cells requires IKK and the mitogen-activated protein kinase p38. They demonstrated that PKB signalling in response to IL-1β exposure stimulates NF-κB by activating p38 in a manner dependent on IKK and that activated p38 indirectly stimulates the transactivation domain of the p65 subunit of NF-κB through a functional interaction with the transcriptional coactivator CREB-binding protein/p300 (CBP/p300). These findings are corroborated by other reports, which show that p38 modulates NF-κB by an indirect mechanism (Wesselborg *et al.*, 1997; Norris & Baldwin, 1999).

One of the main physiological substrates of PKB is glycogen synthase kinase-3 (GSK3), which was discovered over twenty years ago as one of several protein kinases that phosphorylate and inactivate glycogen synthase, the final enzyme in glycogen biosynthesis (Embi *et al.*, 1980). However, GSK3 has now been found to regulate the functions of many metabolic, signalling and structural proteins in a variety of tissues and could be involved in the coordinated regulation of a large number of cellular processes (Grimes & Jope, 2001). Mammalian GSK3 exists as two closely related isoforms termed α and β, each encoded by a distinct gene (Woodgett, 1990, 1991). These proteins share 85% homology at the amino acid level. There is growing evidence that GSK3, particularly the β isoform, is involved in the regulation of gene transcription. An example of this is the phosphorylation of the transcription factor c-Jun, which is a component of the AP-1 transcription factor complex. c-Jun is phosphorylated *in vitro* by GSK3 on three sites that are highly phosphorylated in resting cells and are specifically dephosphorylated when cells are stimulated by growth factors. These sites lie close to the DNA-binding domain of c-Jun and phosphorylation results in a reduction in

DNA-binding affinity (Woodgett, 1991; Plyte *et al.*, 1992). Evidence that GSK3 plays an important role in transcriptional regulation in intact cells comes from observations that transactivation of AP-1 reporter genes is inhibited by coexpression of GSK3 (DeGroot *et al.*, 1992). GSK3 has also been implicated in the control of the transcription factor cyclic-AMP response element binding protein (CREB) by cyclic-AMP and *in vitro* it phosphorylates sites in the transcription factor c-Myc that are phosphorylated *in vivo* (Fiol *et al.*, 1994; DeGroot *et al.*, 1992). More recently it has been reported that GSK3 also plays a critical role in cell survival. Pap & Cooper (1998), demonstrated that overexpression of GSK3 was sufficient to induce apoptosis in Rat-1 and PC12 cells. They suggest that the phosphorylation of one or more targets of GSK3 serve to activate apoptotic cell death alongside other proapoptotic targets of PI3K/PKB signalling such as BAD.

It has been established that inhibition of GSK3 activity *in vivo* results from its phosphorylation at an N-terminal serine residue, Ser-21 in GSK3α and Ser-9 in GSK3β, by PKB (Cross *et al.*, 1995). *In vitro* these residues can also be phosphorylated by MAPK pathways (Sutherland *et al.*, 1993). Given that phosphorylation by GSK3 appears to inhibit the activities of proteins involved in a wide range of biosynthetic processes, inactivation of GSK3 via PI3K/PKB or MAPK may provide a coordinated mechanism for activating a spectrum of cellular activities. PI3K signalling, via PKB and GSK3, appears to trigger a diverse array of cellular responses. Of particular interest is the role of these pathways in transcription factor regulation. The fact that the downstream targets of PI3K can activate transcription factors such as AP-1 and NF-κB in a range of cell types suggests that PI3K signalling could be involved in the gene induction of inflammatory mediators such as iNOS and IL-8.

Three separate lines of evidence implicate a role for PI3K signalling in the regulation of iNOS transcription, although the studies suggest that instead of PI3K activation leading to gene induction of iNOS it actually attenuates iNOS expression. For example, Donaldson and colleagues (1996), demonstrated that while IL-1β activated PI3K in renal mesangial cells, pharmacological inhibition of PI3K by wortmannin actually potentiated the IL-1β-induced NO production. In addition, Wright *et al.* (1997), showed that stimulation of the colonic epithelial cell line HT-29 with IL-13 resulted in the inhibition of proinflammatory cytokine-induced iNOS expression and this was reversed upon PI3K inhibition. Finally Park *et al.* (1997), established that PI3K inhibitors enhanced LPS-induced iNOS expression and activity in murine macrophages. Together these data imply that inhibition of PI3K activity may be essential for iNOS induction. The mechanism by which PI3K might regulate expression of iNOS in these systems is not understood, although IL-13 has been shown to inhibit NF-κB activation in some cell models (Manna & Aggarwal, 1998) and this could contribute to the decrease in iNOS expression observed in colonic epithelial cells. However, given that the general consensus is that PI3K is required for NF-κB activation in most settings, it may be that the activation of PI3K in some cases results in the phosphorylation of an unknown protein that interferes with the transcriptional regulation of iNOS (Wright & Ward, 2000). Perhaps the interaction between iNOS and PI3K varies depending on the cell model used and exhibits tissue-specific regulation.

Interleukin-13 (IL-13) is the most recently identified cytokine to join the T helper 2 (Th2) family. It is distantly related to IL-4 and is produced by activated T-lymphocytes (especially Th2 cells), mast cells, basophils, dendritic cells and natural killer (NK) cells. IL-13 has been shown to induce a variety of immunomodulatory functions in a wide

range of cell types, including macrophages, NK cells, fibroblasts, eosinophils, airway smooth muscle cells and endothelial cells, highlighting its pleiotropic activity (reviewed by Brombacher, 2000). It has recently been reported that the immune response to chronic *Pseudomonas aeruginosa* lung infection in CF patients is predominantly of the Th2 type (Moser *et al.*, 2000). If this is the case there may be high levels of IL-13 in the CF infected lung, which could affect cytokine-induced iNOS expression.

IL-13 activation of a PI3K-dependent signalling cascade has also been linked to the inhibition of proinflammatory cytokine-induced apoptosis in the colonic epithelial cell line HT-29 (Wright *et al.*, 1999). It has been suggested that antiapoptotic genes may be downstream targets of NF-κB (Hsu *et al.*, 1996; Antwerp *et al.*, 1996). If this is the case, PI3K may be involved in protecting cells from apoptosis via NF-κB-dependent mechanisms. However, Wright and colleagues (1999) demonstrated that cytokine-induced apoptosis in HT-29 cells was independent of NO formation. This seems to rule out a link with NF-κB. It is possible that cell survival in this case was a result of the phosphorylation of proapoptotic downstream targets of PKB, including BAD, caspase 9 and FKHRL1 and/or the dephosphorylation of proapoptotic targets of GSK3.

As discussed earlier, there is evidence to suggest that CF airway epithelia show decreased levels of cytokine-induced NO production compared to non-CF airway epithelia (Light *et al.*, 1989; Grasemann *et al.*, 1997; Kelley & Drumm, 1998). It has also been suggested that apoptotic pathways may be altered in CF (Maiuri, 1997). By analogy with colonic epithelial cells it seems possible that PI3K activity could be aberrant in CF airway epithelium. An overactive PI3K system could account for some of the lung manifestations seen in CF.

1.6 AIM OF THE STUDY

This study is based on the finding that activation of phosphatidylinositol 3-kinase (PI3K) leads to the inhibition of iNOS transcription in gut epithelial cells. CF airway epithelia show aberrant production of inflammatory mediators such as nitric oxide and IL-8, which may be a result of altered intracellular signalling. In this study the regulation of the PI3K signal transduction pathway was explored in well characterised airway epithelial cell lines and a possible link with nitric oxide and IL-8 production was investigated.

The objectives of the investigation were:

1. To explore the potential of human airway epithelial cells to produce nitric oxide and IL-8 in response to proinflammatory cytokines.

2. To investigate possible signalling mechanisms involved in the production and regulation of nitric oxide and IL-8 in human airway epithelial cells.

3. To characterise the regulation of the phosphatidylinositol 3-kinase signal transduction pathway in human airway epithelial cells.

The elucidation of the nature of the dysregulated inflammatory response in CF airway epithelia will help in the development of new therapeutic strategies for the effective treatment of this disease.

2 MATERIALS AND METHODS

2.1 MATERIALS

Reagents were purchased from Sigma Chemical Co. (Poole, Dorset, UK), unless otherwise stated.

All cell culture plastics were purchased from Nunc (Nottingham, UK).

All cell culture reagents (media, PBS, HEPES, trypsin/EDTA, foetal calf serum, antibiotics, vitrogen) were purchased from Gibco BRL Life Technologies, Inc. (Paisley, Scotland).

Human recombinant IL-1β was a generous gift from Glaxo Wellcome (Greenford, UK).

Human recombinant TNFα was a generous gift from Bayer (Slough, UK).

Human recombinant IFNγ was purchased from Boehringer Mannheim (Mannheim, Germany).

Human recombinant IL-13 was kindly donated by Dr. A. Minty (Sanofi Elf Bio Recherches, Laberges, France).

All cytokines were diluted in sterile PBS + 0.1% bovine serum albumin (BSA, low endotoxin) and stored in aliquots at -20°C.

Hygromycin, L-arginine, SP600125 and Ro-32-0432 were purchased from Calbiochem (Darmstadt, Germany).

2.2 CELL CULTURE CONDITIONS

2.2.1 9HTEo$^-$ and ΣCFTE29o$^-$ Airway Epithelial Cell Lines

The human tracheal epithelial cell line 9HTEo$^-$ and the human CF tracheal epithelial cell line ΣCFTE29o$^-$ were generous gifts from D.C. Gruenert (University of San Francisco, California). The cell lines were created by transfecting human tracheal epithelial cells with a plasmid containing a replication-defective simian virus 40 (SV40) genome. The process resulted in transformed human tracheal epithelial cells, which retained some aspects of the original phenotype. These include the presence of microvilli, responsiveness to agonists, Cl^- ion transport and the ability to form tight junctions (Gruenert *et al.*, 1988; Knuzelmann *et al.*, 1993).

2.2.2 9HTEo$^-$/pCEPRF and 9HTEo$^-$/pCEP#2 Airway Epithelial Cell Lines

The human tracheal epithelial cell lines 9HTEo$^-$/pCEPRF and 9HTEo$^-$/pCEP#2, were a kind gift from Pam Davis (Case Western, Cleveland, Ohio). The 9HTEo$^-$/pCEPRF cell line was made by transfecting the SV40 transformed cell line 9HTEo$^-$ with a pCEP4 plasmid, containing genes that expressed the regulatory (R) domain of CFTR (Perez *et al.*, 1996). Over expression of the R domain in these cells produced a CF phenotype. The 9HTEo$^-$/pCEP#2 cell line was created in the same way as the 9HTEo$^-$/pCEPRF cell line (Perez *et al.*, 1996). However, in this case the 9HTEo$^-$ cells were transfected with an empty pCEP4 plasmid. The pCEP4 plasmid also contained genes conferring hygromycin B resistance to the pCEP cell lines. The inclusion of hygromycin B in the cell culture medium ensured that only cells containing the pCEP4 plasmid would survive and replicate.

2.2.3 A549 Airway Epithelial Cell Line

The human type II alveolar carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC).

2.2.4 Cell Culture Vessels

All cell culture vessels used were coated with a solution of 6.6% collagen (1 ml vitrogen, 13 ml sterile Milli-Q water, 1 ml serum-free medium, 0.1 M NaOH). Once the collagen solution had been added to the vessels the excess was removed and the vessels were left to dry in the tissue culture hood overnight. Coated vessels were stored at 4°C until use.

2.2.5 Cell Culture

All solutions used for cell culture were prepared using sterile Milli-Q water obtained from a Milli-Q PF Plus system with ultrafiltration cartridge (Millipore UK Ltd).

9HTEo$^-$ cells and ΣCFTE29o$^-$ cells were routinely cultured in 180 cm^2 tissue culture flasks in Eagle's minimum essential medium (MEM) supplemented with penicillin (10 u/ml), streptomycin (10 μg/ml), fungizone (0.5 μg/ml) and 10% (v/v) foetal calf serum (FCS) (referred to as complete medium). Cultures were maintained at 37°C in a humidified air / 5% CO_2 atmosphere. The medium was changed every 2-3 days.

9HTEo$^-$/pCEP#2 cells and 9HTEo$^-$/pCEPRF cells were routinely cultured in 180 cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium with sodium pyruvate + L-glucose + pyridoxine (DMEM). This medium was supplemented with penicillin (10

u/ml), streptomycin (10 μg/ml), fungizone (0.5 μg/ml), hygromycin (10 μg/ml) and 10% (v/v) FCS (referred to as complete medium). Cultures were maintained at 37°C in a humidified air / 5% CO_2 atmosphere. The medium was changed every 2-3 days.

A549 cells were routinely cultured in 80cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium with sodium pyruvate + L-glucose + pyridoxine (DMEM). This medium was supplemented with penicillin (10 u/ml), streptomycin (10 μg/ml), fungizone (0.5 μg/ml) and 10% (v/v) FCS (referred to as complete medium). Cultures were maintained at 37°C in a humidified air / 5% CO_2 atmosphere. The medium was changed every 2-3 days.

To subculture confluent monolayers, the medium was removed and the cells were washed. 9HTEo$^-$ cells, ΣCFTE29o$^-$ cells and A549 cells were washed 3 times with PBS (w/o Ca^{2+} and Mg^{2+}). 9HTEo$^-$/pCEP#2 cells and 9HTEo$^-$/pCEPRF cells were washed 3 times with PBS (w/o Ca^{2+} and Mg^{2+}) + 20 mM HEPES. After washing 1.5 ml of 0.05% (w/v) trypsin and 0.02% (w/v) EDTA were added to each flask and the cells were incubated for approximately 3 minutes at 37°C until the cells had detached from the flask. The action of trypsin/EDTA was inhibited by the addition of 10 ml of complete medium and the cell suspension was centrifuged at 300 g for 10 minutes. The cell pellet was resuspended in 1 ml of complete medium and cell number and viability were checked in a Neubauer haemocytometer after mixing 1:1 with 0.4% Trypan Blue. Dead cells stained blue due to the uptake of Trypan blue. Cell viability was always greater than 90%. Cells were counted and then seeded at 3-5 x 10^4 cells/ml of complete medium, into 180 cm^2 tissue culture flasks (25 ml medium per flask) for further culture, or into 6-well plates (2 ml medium per well) or

petri dishes (4 ml medium per dish) for experimental protocols. Flasks, plates and dishes reached confluency after approximately 5 days.

For storage, cells were resuspended at 1 x 10^6 cells/ml of freeze medium. The freeze medium contained 10% dimethylsulphoxide (DMSO), 40% FCS and 50% complete medium. The cell suspension was transferred to cryotubes at 1ml/ tube. The cryotubes were gradually cooled in isopropyl alcohol at –70°C overnight and then stored in liquid nitrogen tanks. For resuscitation from liquid nitrogen, cells were rapidly defrosted at 37°C in a water bath, washed in complete medium and resuspended in 1 ml of complete medium. Cells from one cryotube were seeded into 180 cm^2 tissue culture flasks in complete medium, continuing as above.

2.3 EXPERIMENTAL PROTOCOL

For experimental purposes cells were grown until they were approximately 90% confluent. Four to twelve hours prior to the experiment the monolayers were washed in PBS (w/o Ca^{2+} and Mg^{2+}) or PBS (w/o Ca^{2+} and Mg^{2+}) + 20 mM HEPES and cultured in FCS-free medium (MEM or DMEM). The growth arrested cultures were then stimulated with the appropriate doses of cytokines, drugs or vehicle controls for the times detailed in the results section. Supernatants were collected from the 6-well plates, centrifuged to remove cellular debris and either assayed for nitrite or stored at –20°C until assayed for IL-8. Cells were detached from the base of the wells by adding 1.5 ml of 0.05% (w/v) trypsin and 0.02% (w/v) EDTA and incubating for approximately 3 minutes at 37°C. Cell number was determined in a Neubauer haemocytometer after mixing 1:1 with 0.4% Trypan blue and the total amount of nitrite and IL-8 produced was expressed per 10^6 cells. Total cellular protein was extracted from the cells growing in petri dishes (see 2.6.1) for analysis via immunoblotting.

2.4 FLUOROMETRIC NITRITE ASSAY

Nitric oxide (NO) concentrations in culture supernatants were determined by measuring nitrite, the stable end product of nitric oxide breakdown. All samples were measured in duplicate. Nitrite was measured using a fluorometric assay based upon the reaction of 2,3-diaminonaphthalene (DAN, Lancaster Synthesis Ltd, Morecombe, UK) with nitrite under acidic conditions to form the fluorescent product 1-(H)-naphthotriazole. The assay was modified from the method of Misko and colleagues (1993), for use on a Photon Technology International (PTI) spectrofluorimeter. As specified by Misko *et al.* (1993), an excitation wavelength of 365 nm and an emission wavelength of 405 nm were used. A standard curve of sodium nitrite in FCS-free medium ranging from 20 nM to 3.0 μM was prepared. The standards also contained the same percentage of vehicle as the culture supernatants. The reaction was initiated by mixing 1 ml of standard or culture supernatant with 100 μl of freshly prepared DAN reagent (50 μg/ml DAN in 0.62 M HCl) in disposable cuvettes. The mixtures were incubated at room temperature in the dark for 10 minutes and the reaction was stopped by the addition of 1 ml of 0.28 M NaOH. The samples were read in the spectrofluorimeter and the nitrite concentration was calculated by reference to the standards. Phenol red present in both MEM and DMEM did not interfere with the assay. The sensitivity of the assay was 20 nM.

2.5 INTERLEUKIN-8 ENZYME-LINKED IMMUNOSORBENT ASSAY (IL-8 ELISA)

Extracellular IL-8 concentration of culture supernatants was measured by double ligand ELISA. All samples were measured in duplicate. 96 well microtitre plates (immuno maxisorb plates) were coated with 100 μl per well of mouse monoclonal

anti-human IL-8 antibody (1 μg/ml, a generous gift from Novartis, Vienna, Austria) in carbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3), pH 9.6. Plates were covered, incubated overnight at 4°C and then washed three times in wash buffer (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2HPO_4, 8.1 mM Na_2HPO_4, 0.05% (v/v) Tween-20), pH 7.3. Plates were blocked by the addition of 100 μl per well of 5% foetal bovine serum (FBS) in coating buffer and left for 1 hour at room temperature. Three more washes followed. A range of IL-8 standards were prepared (0.1 -10 ng/ml) in dilution buffer (wash buffer plus 2% FBS) from a 10 μg/ml stock solution (R&D Systems Inc., Abingdon, UK) and the culture supernatants were appropriately diluted in the same buffer. Standards and samples (50 μl per well) were added in duplicate to the plates and incubated for 2 hours at 37°C. Plates were washed 3 times and 50 μl per well of biotinylated goat polyclonal anti-human IL-8 detecting antibody (0.5 μg/ml, a generous gift from Novartis, Vienna, Austria) in dilution buffer was added to the plates for 1 hour at 37°C. Three more washes followed and 50 μl per well of streptavidin peroxidase (0.5 μg/ml, R&D Systems Inc., Abingdon, UK) in dilution buffer was added to the plates for 30 minutes at 37°C. Plates were washed three times. One 10 mg tablet of 1,2 O-phenylene dihydrochloride (OPD) and 20 μl of 30% hydrogen peroxide were added to 50 ml of warmed substrate buffer (34 mM citric acid monohydrate, 66 mM Na_2HPO_4), pH 5.0. This solution was added to the plates (100 μl per well), which were incubated for 30 minutes at room temperature in the dark. The reaction was terminated with 150 μl per well 1 M H_2SO_4 and the OD measured at 490 nm.

2.6 IMMUNOBLOT ANALYSIS

Growth arrested cell monolayers were incubated with appropriate stimuli and proteins forming part of the PI3K signalling pathway were extracted for analysis.

2.6.1 Preparation of Whole Cell Extracts

After removal of culture supernatants, the cells were washed 3 times in PBS/PBS + 20 mM HEPES and the cell culture vessels were placed on ice. Ice-cold lysis buffer including protease inhibitors (0.4 ml) (see appendix), was added to each sample and the samples were thoroughly homogenised. The whole cell extracts were then transferred to cold sterile 1.5 ml centrifuge tubes and rotated for 15 minutes at 4°C. The samples were centrifuged at 12,000 g for 2-3 minutes to pellet the debris. Twenty µl of cleared lysate was removed from each sample for protein quantification and150 µl of lysate was transferred to fresh sterile 1.5 ml centrifuge tubes along with 30 µl of 5X sample buffer (see appendix). The samples were then placed in a heating block at 85-90°C for 15 minutes, cooled on ice and stored at –70°C.

2.6.2 Quantification of Protein

The protein assay was based on the Bradford procedure (Bradford *et al.*, 1976). A 1 mg/ml solution of bovine serum albumin (BSA) was prepared in PBS (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2HPO_4, 8.1 mM Na_2HPO_4), pH 7.4 and used to produce known concentrations of BSA in 1 ml of Bio-Rad protein Assay (Bio-Rad, Munchen, Germany). Equal volumes of each sample (20 µl) were added to 980 µl of Bio-Rad protein Assay and 200 µl of diluted sample or standard were transferred in duplicate to the wells of a 96-well microtitre plate. Protein concentration was measured with a microplate reader at 595 nm.

2.6.3 Separation of Cellular Proteins

Proteins were analysed by one dimensional gel electrophoresis, which under reducing conditions separates proteins based on molecule size. Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli *et al.* (1970), using the Protean II xi gel system (Bio-Rad, Munchen, Germany). Acrylamide running gels (7.5% w/v) were prepared (see appendix), poured into the gel equipment and overlaid with distilled water. After 1 hour the water was removed and a 5% w/v acrylamide stacking gel (see appendix) was poured on top of each running gel and set with a 10-lane comb. Once set the gels were transferred to a tank filled with electrophoresis buffer (25 mM TRIS, 192 mM glycine, 0.1% SDS) and the combs were removed. The gels were loaded with approximately 30 μg of sample per lane and a prestained, broad range, molecular weight marker (Bio-Rad, Munchen, Germany) was included on each gel. The gels were run at room temperature at 50 V until the bromophenol blue tracking dye entered the running gel. The voltage was then increased to 150 V. Gels were run until the bromophenol blue had reached the bottom of the resolving gel. The gels were then placed in transfer buffer (25 mM TRIS, 192 mM glycine, 20% methanol) in preparation for blotting.

2.6.4 Electrophoretic Transfer of Proteins to Nitro-cellulose

The separated proteins were transferred to nitro-cellulose membrane using a transblot electrophoretic apparatus (Bio-Rad, Munchen, Germany). Each polyacrylamide gel was soaked for 10 minutes in transfer buffer and the lanes of the stacking gel were removed. Four pieces of filter paper, a piece of nitro-cellulose membrane, just slightly larger than the gel and two nylon pads were also soaked in transfer buffer. A sandwich was constructed in which each gel was overlaid with a

piece of wet nitro-cellulose membrane (Boehringer Mannheim, Mannheim, Germany), taking care to remove all air bubbles and these were surrounded on both sides by two layers of filter paper (Whatman, Maidstone, Kent, UK) and a nylon pad. This sandwich was enclosed in a cassette and placed in a transfer tank (Bio-Rad, Munchen, Germany) with the nitro-cellulose nearest the anode. The tank was filled with cold transfer buffer and electrophoretic transfer was carried out at 100 V for 1 hour at 4°C. To check for successful protein transfer each piece of nitro-cellulose membrane was soaked in Ponceau. To remove the Ponceau, the membranes were washed with TBS/Tween (50 mM TRIS, 150 mM NaCl, 0.1% (v/v) Tween-20), pH 7.6 for a few minutes.

2.6.5 Immunoblotting of Nitro-Cellulose–Bound Protein

Immunoblot analyses of phospho-PKB/Akt, phospho-GSK3 and PTEN were performed as described in specific Cell Signalling Technology Anitbody Kits: PhosphoPlus Akt (Ser473), PhosphoPlus GSK3α/β (Ser21/9) and PTEN respectively (Cell Signalling Technology, Hertfordshire, UK). All the following steps were carried out with gentle shaking. Non-specific binding sites on the nitro-cellulose were blocked by incubating the membranes for 1 hour in 5% non-fat dried milk protein reconstituted in TBS/Tween. The membranes had three 5 minute washes in TBS/Tween and were then incubated overnight with rabbit anti-human phospho-specific Akt (Ser473), rabbit anti-human phospho-specific GSK3α/β (Ser21/9) or rabbit anti-human PTEN diluted 1:1000 in TBS/Tween. The membranes had three further 5 minute washes in TBS/Tween before a 1 hour incubation with anti-rabbit secondary antibody conjugated to horseradish peroxidase (DAKO, Glostrup, Denmark), diluted 1:2000 in TBS/Tween. After further washing in TBS/Tween (3 x 5 min) phospho-Akt, phospho-GSK3α/β or PTEN were detected by

incubating each membrane with 10 ml LumiGLO (Amersham Pharmacia, Buckinghamshire, UK) for 1 minute at room temperature. The membranes were drained of excess developing solution, wrapped in cling film and exposed to Kodak omat AR5 X-ray film for an appropriate time. To verify equal loading of protein, additional gels were run using the same samples and the resulting nitro-cellulose membranes probed for non-phospho PKB/Akt, using the same conditions as previously described. In order to help analyse the immunoblot data, densitometry analysis was carried out. For each blot, the density of the darkest band was measured and normalised to 100%. The relative densities of the other bands were calculated accordingly.

2.7 IN VITRO LIPID KINASE ASSAY

2.7.1 Cell Stimulation

Growth arrested cultures were washed 3 times in PBS and 5 ml of Versene (1:5000, Invitrogen Ltd, California, USA) was added for 5 minutes at 37°C. FCS-free medium (10 ml) was then added and the cell suspension was centrifuged at 300 g for 10 minutes. The cell pellet was resuspended in FCS-free medium at 1×10^7 cells/ml and aliquoted into 1.5 ml centrifuge tubes at 5×10^6 cells/sample. The samples were then equilibrated for 15 minutes at 37°C. If used, the cells were pre-incubated with wortmannin or vehicle control for 15 minutes. Cells were stimulated for appropriate times with agonist and the reactions were terminated by pulsing the cells for 6 seconds in a microcentrifuge, removing the supernatant and adding 500 µl of ice-cold lysis buffer (see appendix). The samples were incubated on a rotator for 10 minutes at 4°C and any non-soluble material was removed by spinning in a microcentrifuge at 12,000 g for 10 minutes.

2.7.2 Immunoprecipitation of Phosphatidylinositol 3-Kinase

Protein-A sepharose beads were prepared by mixing a known volume of beads with an equal volume of lysis buffer (minus protease inhibitors). After 30 minutes the beads were pulsed in a microcentrifuge for 15 seconds and the supernatant was removed. The beads were washed a further 3 times in lysis buffer (minus inhibitors) and finally resuspended in enough lysis buffer (minus inhibitors) to form a 50:50 slurry. The cell lysates were incubated overnight with 4 μg of mouse monoclonal anti-PI3K p85 antibody (Upstate Biotechnology, Milton Keynes, UK) on a rotator at 4°C. The immunocomplex was then captured by adding 50 μl of prepared Protein-A sepharose to each sample and incubating for a further 2 hours on a rotator at 4°C. Finally the samples were pulsed for 15 seconds in a microcentrifuge to pellet the beads and the supernatant was removed and discarded.

2.7.3 Phosphatidylinositol 3-Kinase Assay

PI3K assays were carried out following the procedure described by Gold *et al.* (1992), with some modifications. The immunoprecipitates were washed 3 times in wash buffer A (PBS, 1% NP-40, 100 μM Na_3VO_4), 3 times in wash buffer B (100 mM TRIS, pH 7.4; 5 mM LiCl, 100 μM Na_3VO_4) and 3 times in wash buffer C (10 mM TRIS, pH 7.4; 150 mM NaCl, 5 mM EDTA, 100 μM Na_3VO_4). Between each wash, the beads were pelleted by pulsing at 12,000 g for 5 seconds in a microcentrifuge. The final wash was removed as completely as possible and 50 μl of wash buffer C, 20 μg of phosphatidylinositol and 10 μl of 100 mM $MgCl_2$ was added to each pellet. The kinase reactions were started by adding 5 μl of ATP reaction buffer (0.88 mM ATP, 20 mM $MgCl_2$, 30 μCi [γ^{32}P]-ATP [Amersham Pharmacia, Buckinghamshire, UK]) per sample. The reactions were allowed to proceed for 10 minutes at room

temperature and were terminated by the addition of 20 µl of 5 M HCl and 160 µl of chloroform/methanol (1:1). The phases were mixed by vortexing and separated by centrifuging at 12,000 g for 10 minutes. Fifty µl of the lower, chloroform phase was removed and spotted onto oxalate-treated thin-layer chromatography (TLC) plates (BDH, Poole, Dorset). The lipids were resolved by thin-layer chromatography in a mixture of chloroform : methanol : water : ammonium hydroxide (60:47:11.3:2). Once resolved, the [^{32}P]-labelled lipids were visualised by wrapping the TLC plate in cling film and exposing it to Kodak omat AR5 X-ray film at –70°C for an appropriate time.

2.8 IN VITRO LIPID PHOSPHATASE ASSAY

2.8.1 Preparation of Whole Cell Extracts

Growth arrested cultures were washed 3 times in PBS and cells were removed from the tissue culture flask using Versene (1:5000, Invitrogen Ltd, California, USA) and resuspended in FCS-free medium, as previously described. The cell pellet was resuspended in FCS-free medium at 1 x 10^7 cells/ml and aliquoted into 1.5 ml centrifuge tubes at 5 x 10^6 cells/sample. The cells were pulsed for 6 seconds in a microcentrifuge and the supernatant removed. Ice-cold lysis buffer (500 µl) (see appendix) was added to each pellet. The samples were incubated on a rotator for 10 minutes at 4°C and any non-soluble material was removed by spinning in a microcentrifuge at 12,000 g for 10 minutes.

2.8.2 Immunoprecipitation of PTEN

Protein-A sepharose beads were prepared as previously described. The cell lysates were incubated overnight with a 1:50 dilution of rabbit polyclonal anti-PTEN antibody (Cell Signaling Technology, Hertfordshire, UK) on a rotator at 4°C. The immunocomplex was then captured by adding 50 µl of prepared Protein-A sepharose to each sample as previously described.

2.8.3 Phosphatase Assay

The immunoprecipitates were washed 3 times in lysis buffer (minus inhibitors). Between each wash, the beads were pelleted by pulsing at 12,000 g for 5 seconds in a microcentrifuge. The final wash was removed as completely as possible and 25 µl of assay buffer (500 mM TRIS, pH 7.5; 10 mM $MgCl_2$) was added to each sample along with 3 µl of [^{3}H]-PtdIns-1,3,4,5-P_4 (Amersham Pharmacia, Buckinghamshire, UK). The reactions were allowed to proceed for 20 minutes at 37°C and were terminated by the addition of 150 µl of acidified chloroform/methanol (1:3) to each sample. The samples were mixed by vortexing and 50 µl of chloroform plus 50 µl of 10 mM HCl were added followed by centrifugation at 12,000 g for 5 minutes. The aqueous phase was removed into clean centrifuge tubes and dried *in vacuo* for 1 hour. After drying each pellet was resuspended in 100 µl of filtered Milli-Q water and vortexed.

2.8.4 High Pressure Liquid Chromatography (HPLC) Analysis

The products of the reaction were separated on an HPLC column (12.5cm Whatman Partisphere SAX column, Maidstone, Kent, UK). They were eluted from the column

using a gradient comprising Milli-Q water and phosphate buffer (1.25 M NH_4HPO_4), adjusted to pH 3.8 with H_3PO_4. All solutions used on the HPLC column were filtered (0.2 μm Whatman filter paper, Maidstone, Kent, UK) and degassed with helium for approximately 10 minutes. The gradient flow rate in the column was 1 ml/minute and the total elution time was 90 minutes per sample. As the reaction products eluted they were collected in scintillation vials. 3 ml of scintillation fluid was added to each vial and the [^{3}H]-labelled lipids were detected via scintillation counting.

2.9 STATISTICAL ANALYSIS

Duplicate determinations were performed in each experiment. The number (n) of independent experiments is given in the respective figure legend. Data were analysed by two-way analysis of variance (ANOVA) to determine if any statistical significance existed within the data groups. ANOVA was followed by Dunnett's test for the comparison of multiple groups to controls. Data were expressed as means ± SEM of n independent experiments. A probability value of $p<0.05$ was taken as the criterion for a significant difference.

3 NITRIC OXIDE PRODUCTION BY AIRWAY EPITHELIAL CELLS

3.1 INTRODUCTION

Initial investigations sought to examine the mechanisms involved in the production and regulation of nitric oxide (NO) by CF and non-CF human airway epithelial cells in response to proinflammatory cytokine stimulation, using the 9HTEo$^-$, ΣCFTE29o$^-$, 9HTEo$^-$/pCEP#2 and 9HTEo$^-$/pCEPRF cell lines as models of human airway epithelium. The 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines are both human tracheal epithelial cells exhibiting a non-CF and a CF phenotype respectivley. As discusssed in the preceding chapter, the pCEP lines are a genetically matched pair of cell lines. The 9HTEo$^-$/pCEPRF cell line was created by transfecting 9HTEo$^-$ cells with a pCEP4 plasmid containing genes that express the regulatory (R) domain of CFTR (Perez *et al.*, 1996). Overexpression of the R domain is thought to block the ion channel rendering CFTR non-functional and producing a CF phenotype. The 9HTEo$^-$/pCEP#2 cell line was created in a similar fashion, but in this case 9HTEo$^-$ cells were transfected with an empty pCEP4 plasmid producing a matched control line (Perez *et al.*, 1996).

As a prelude to the experiments on the 9HTEo$^-$, ΣCFTE29o$^-$, 9HTEo$^-$/pCEP#2 and 9HTEo$^-$/pCEPRF cell lines, it was necessary to establish the biological activity of the proinflammatory cytokines which were to be used in the stimulations. In addition, the suitablility of the fluorometric nitrite assay as a means of measuring cytokine-induced NO production needed to be assessed. To address these issues stimulations were initially carried out on the type II alveolar carcinoma cell line A549. This cell line was considered appropriate as it had previously been demonstrated that the combination of

TNFα, IL-1β and IFNγ lead to a significant increase in the levels of iNOS generated nitrite in A549 cell culture supernatants compared with supernatants from unstimulated cells (Asano *et al.*, 1994; Robbins *et al.*, 1994).

For experimental purposes, cells were seeded into 6-well plates at 3-5 x 10^4 cells/ml in a total volume of 2 ml of complete medium and grown until they were approximately 90% confluent. The cells were then serum starved for 12 hours and subsequently stimulated for 24 hours at 37°C with vehicle or increasing concentrations of IFNγ (0-300 units/ml), IL-1β (0-10 ng/ml) and TNFα (0-100 ng/ml), either alone or in various combinations. Each stimulation also included 1 mM L-arginine to ensure that substrate availability was not a limiting factor for nitric oxide synthase activity. Levels of nitrite (the stable breakdown product of NO) were determined in cell culture supernatants using a fluorescent substrate as described in the previous chapter.

3.2 NITRITE GENERATION BY A549 CELLS

A549 cells produced a constitutive amount of nitrite in the absence of cytokine stimulation (0.73 ± 0.01 μmoles/10^6 cells) (Fig. 8). Stimulation of the cells with the three cytokines combined had the effect of significantly increasing the amount of nitrite generated compared with constiutive levels (Fig. 8) ($p<0.01$). In fact, the highest concentration of cytokines resulted in an approximate 5 fold enhancement of nitrite levels (3.62 ± 0.5 μmoles/10^6 cells). The data from these experiments verified that the cytokines were active and validated the use of the fluorometric nitrite assay for measurement of cytokine-induced NO production.

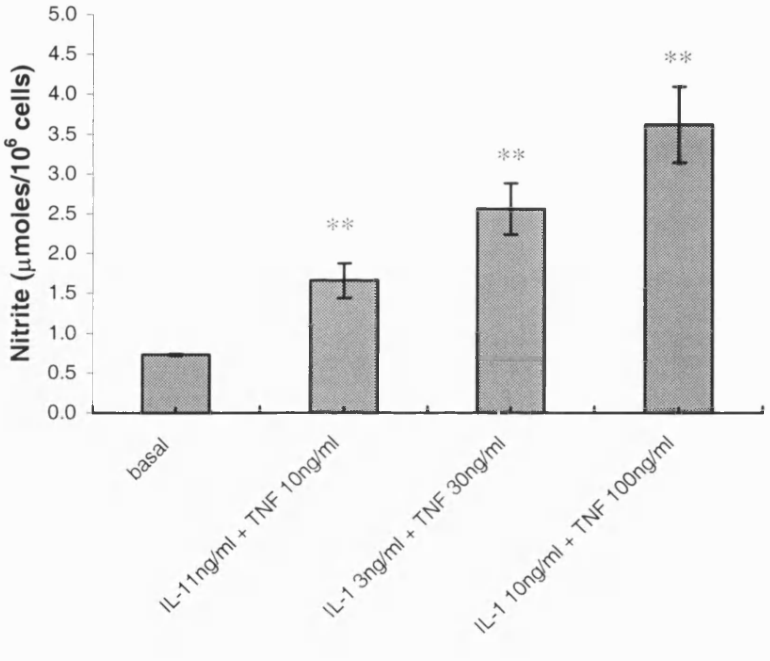


Figure 8 Nitrite production by A549 cells after 24 h incubation at 37°C with IFNγ (100 units/ml), IL-1β (1-10 ng/ml) and TNFα (10-100 ng/ml) added in combination. The cells were serum starved for 12 hours prior to treatment. 1 mM L-arginine was added to each stimulation. Basal is the amount of nitrite produced in the presence of vehicle alone. Each bar is the mean ± SEM of three experiments (** $p<0.01$ compared with basal).

3.3 NITRITE GENERATION BY 9HTEo$^-$ and ΣCFTE29o$^-$ CELLS

Both 9HTEo$^-$ and ΣCFTE29o$^-$ cells produced a constitutive amount of nitrite in the absence of cytokine stimulation (316.23 ± 36.12 nmoles/10^6 cells for 9HTEo$^-$ cells and 778.51 ± 36.43 nmoles/10^6 cells for ΣCFTE29o$^-$ cells), with the ΣCFTE29o$^-$ cell line producing significantly more nitrite than the 9HTEo$^-$ cell line ($p<0.01$) (Fig 9a). Increasing concentrations of the proinflammatory cytokines IFNγ, IL-1β and TNFα, added alone to either cell line, had no effect on nitrite generation compared to the constitutive nitrite production in vehicle treated cells (Figs 9 & 10). Similarly, combinations of IFNγ/IL-1β and IFNγ/TNFα had no effect on the amount of nitrite produced by either cell line (Figs 11 & 12). Stimulation of the cells with high concentrations of the three proinflammatory cytokines combined also had no effect on the amount of nitrite generated compared to constitutive levels (Fig 13).

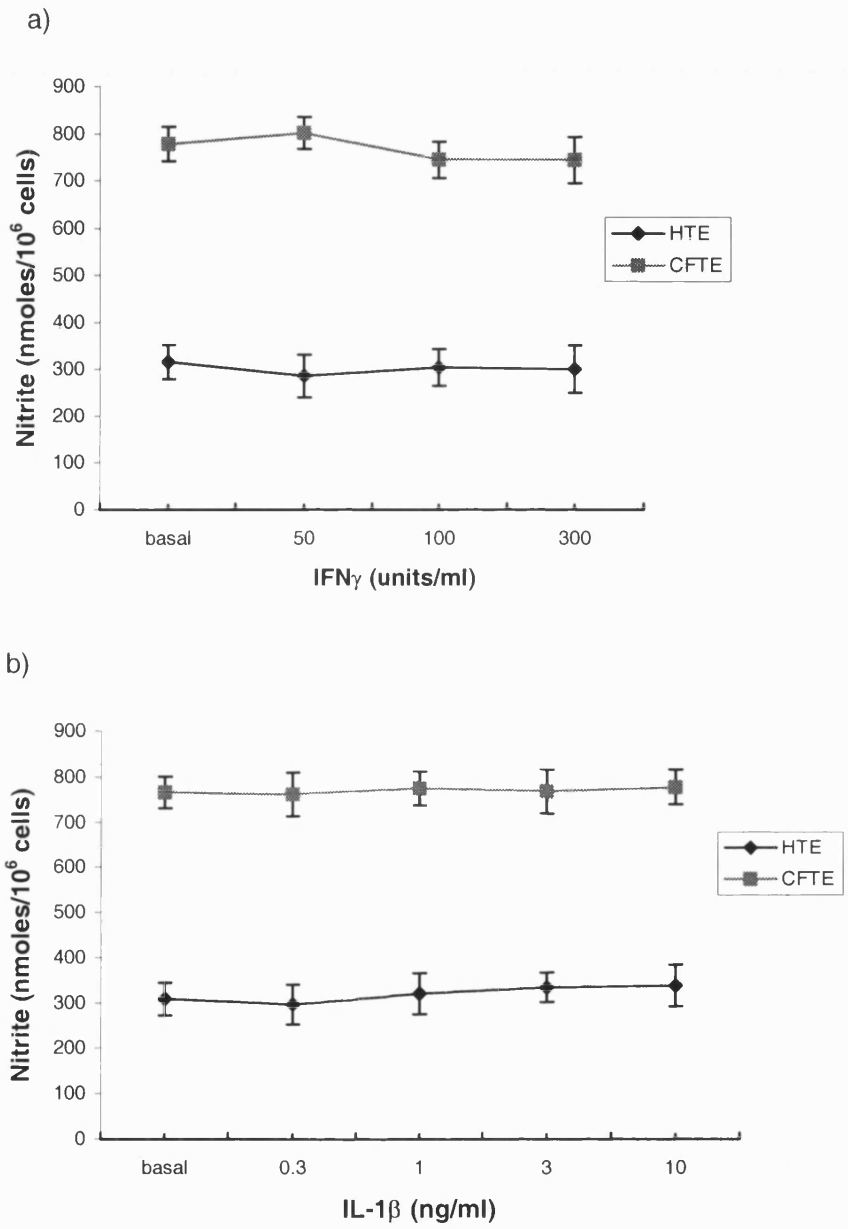


Figure 9 Nitrite production by 9HTEo⁻ and ΣCFTE29o⁻ cells after 24 h treatment at 37°C with (a) increasing concentrations of IFNγ (50-300 units/ml) and (b) increasing concentrations of IL-1β (0.3-10 ng/ml). The cells were serum starved for 12 hours prior to treatment. 1 mM L-arginine was added to each stimulation. Basal is the amount of nitrite produced in the presence of vehicle alone. Each point is the mean ± SEM of three experiments.

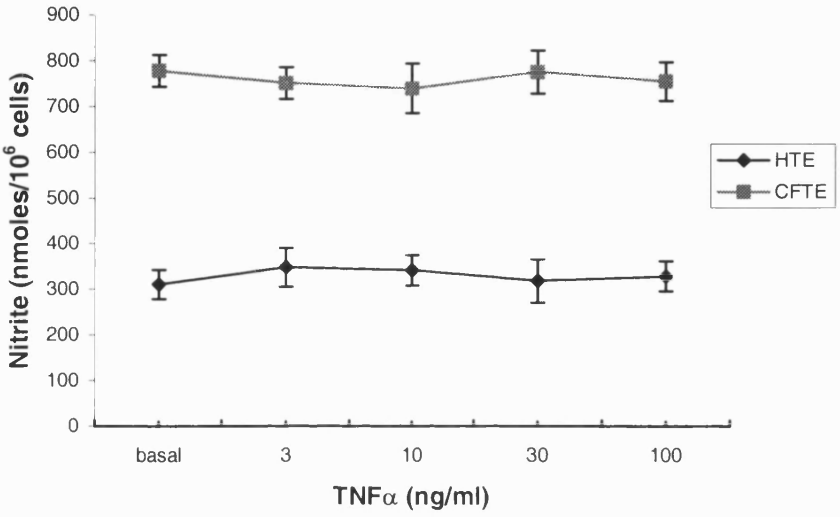
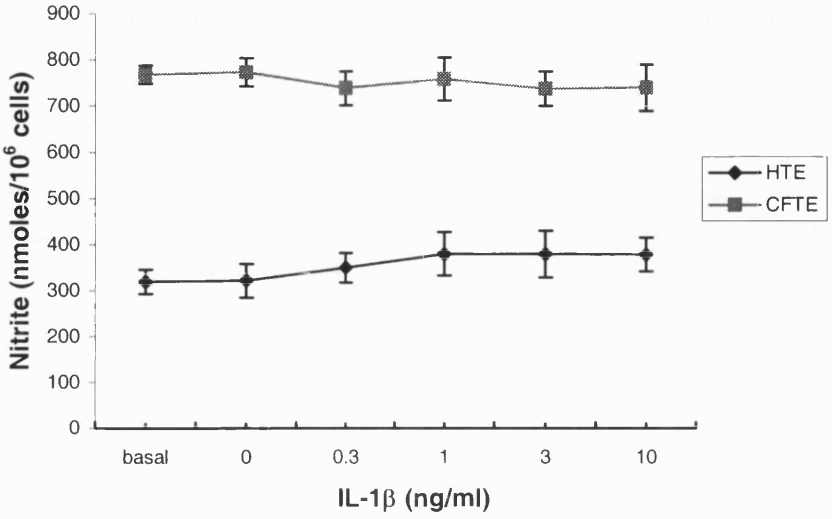


Figure 10 Nitrite production by 9HTEo⁻ and ΣCFTE29o⁻ cells after 24 h treatment at 37°C with increasing concentrations of TNFα (3-100 ng/ml). The cells were serum starved for 12 hours prior to treatment. 1 mM L-arginine was added to each stimulation. Basal is the amount of nitrite produced in the presence of vehicle alone. Each point is the mean ± SEM of three experiments.

a)



b)

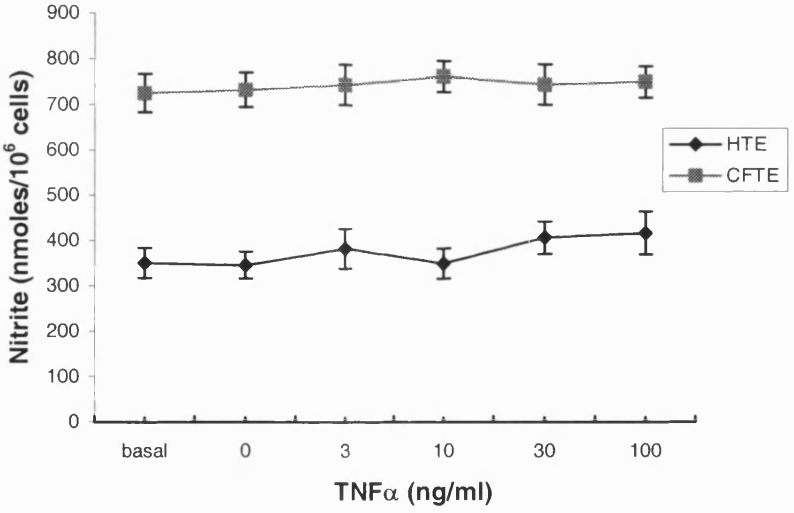


Figure 11 Nitrite production by 9HTEo$^-$ and ΣCFTE29o$^-$ cells after 24 h treatment at 37°C with (a) increasing concentrations of IL-1β (0-10 ng/ml) and (b) increasing concentrations of TNFα (0-100 ng/ml), in the presence of IFNγ 100 units/ml. The cells were serum starved for 12 hours prior to treatment. 1 mM L-arginine was added to each stimulation. Basal is the amount of nitrite produced in the presence of vehicle alone. Each point is the mean ± SEM of three experiments.

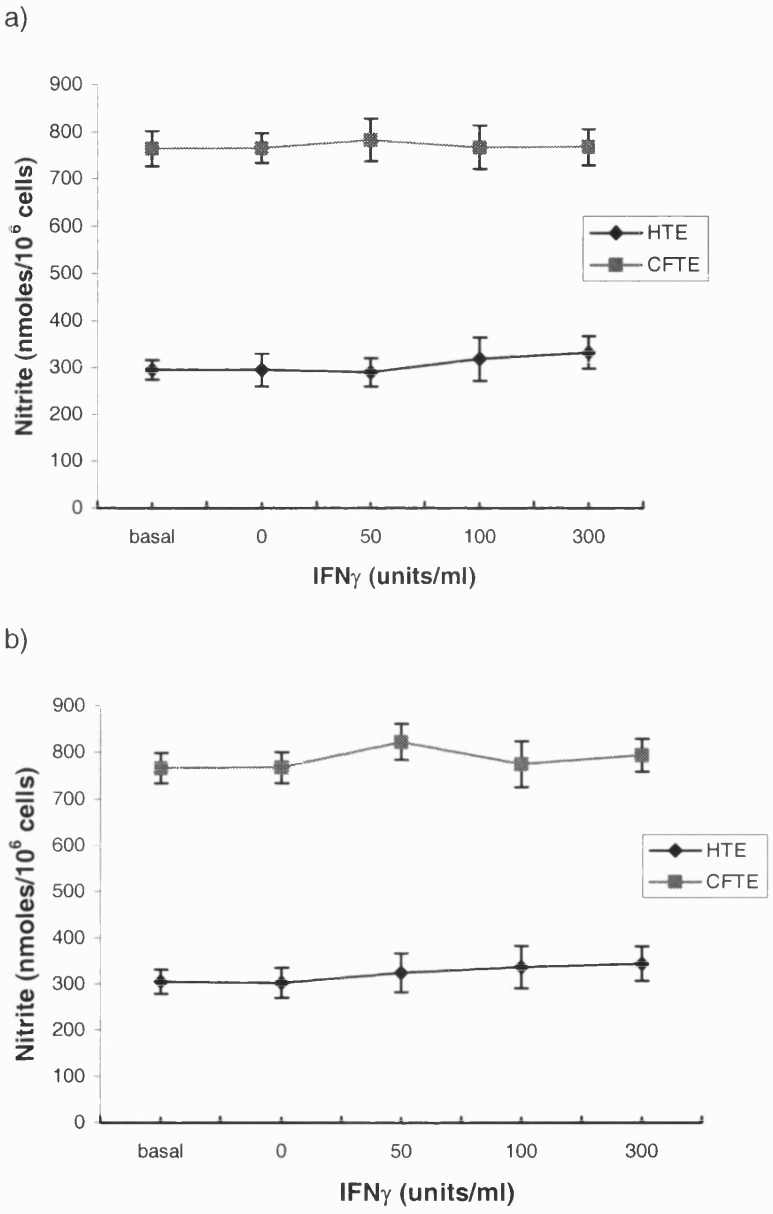


Figure 12 Nitrite production by 9HTEo$^-$ and ΣCFTE29o$^-$ cells after 24 h treatment at 37°C with increasing concentrations of IFNγ (0-300units/ml) in the presence of (a) IL-1β (3 ng/ml) and (b) TNFα (30 ng/ml). The cells were serum starved for 12 hours prior to treatment. 1 mM L-arginine was added to each stimulation. Basal is the amount of nitrite produced in the presence of vehicle alone. Each point is the mean ± SEM of three experiments.

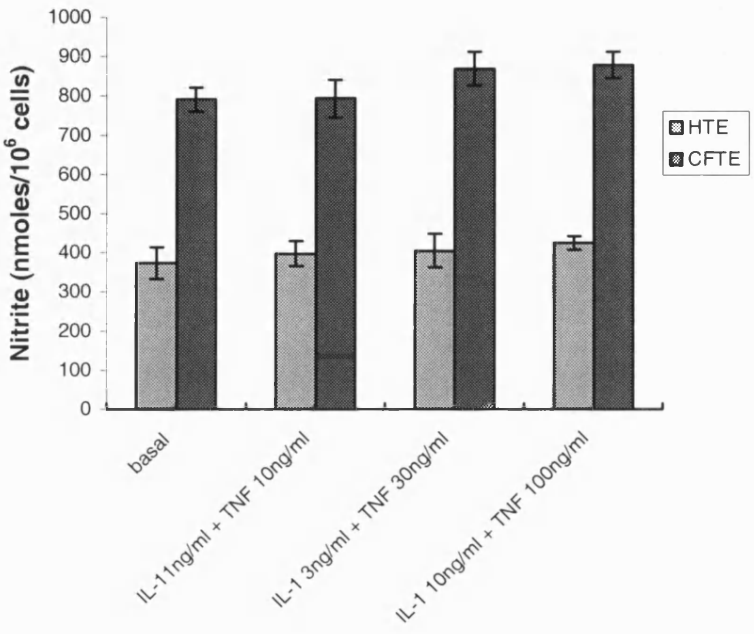


Figure 13 Nitrite production by 9HTEo$^-$ and ΣCFTE29o$^-$ cells after 24 h incubation at 37°C with IFNγ (100 units/ml), IL-1β (1-10 ng/ml) and TNFα (10-100 ng/ml) added in combination. The cells were serum starved for 12 hours prior to treatment. 1 mM L-arginine was added to each stimulation. Basal is the amount of nitrite produced in the presence of vehicle alone. Each bar is the mean ± SEM of three experiments.

Time course studies were carried out to investigate if stimulation of the airway epithelial cells with IFNγ 100 units/ml, IL-1β 3 ng/ml and TNFα 30 ng/ml (cytomix) resulted in induction of nitric oxide at timepoints other than 24 hours. These studies revealed a time-dependent increase in constitutive nitrite levels of 78.49 ± 24.05, 181.05 ± 26.21, 204.26 ± 27.51, 328.43 ± 49.61 and 2733.55 ± 226.05 nmoles/10^6 cells at 6 h, 12 h, 18 h, 24 h and 48 h respectively for the 9HTEo$^-$ cell line and 101.80 ± 16.24, 330.00 ± 38.43, 526.88 ± 38.32, 841.73 ± 45.10 and 3243.44 ± 198.61 nmoles/10^6 cells at 6 h, 12 h, 18 h, 24 h and 48 h respectively for the ΣCFTE29o$^-$ cell line (Fig 14). At each timepoint other than 48 h, the level of nitrite produced by the ΣCFTE29o$^-$ cell line was significantly greater than that produced by the 9HTEo$^-$ cell line ($p<0.01$). Stimulation of either cell line with cytomix did not significanlty increase the levels of nitrite produced at any time point. Interestingly, at 48 h the amount of nitrite in the cell culture supernatant from both cell lines was significantly larger than that produced at other time points ($p<0.01$) in both basal and stimulated conditions. Microscopic examination of cells from this timepoint showed a decrease in cell number and an increase in cell debris in each case. Cell viability at 48 h was examined by trypan blue exclusion and was found to be 55% for 9HTEo$^-$ cells and 46% for ΣCFTE29o$^-$ cells. At all other time points cell viability was always greater than 85%. In view of these data, all subsequent experiments were carried out over a 24-hour time period.

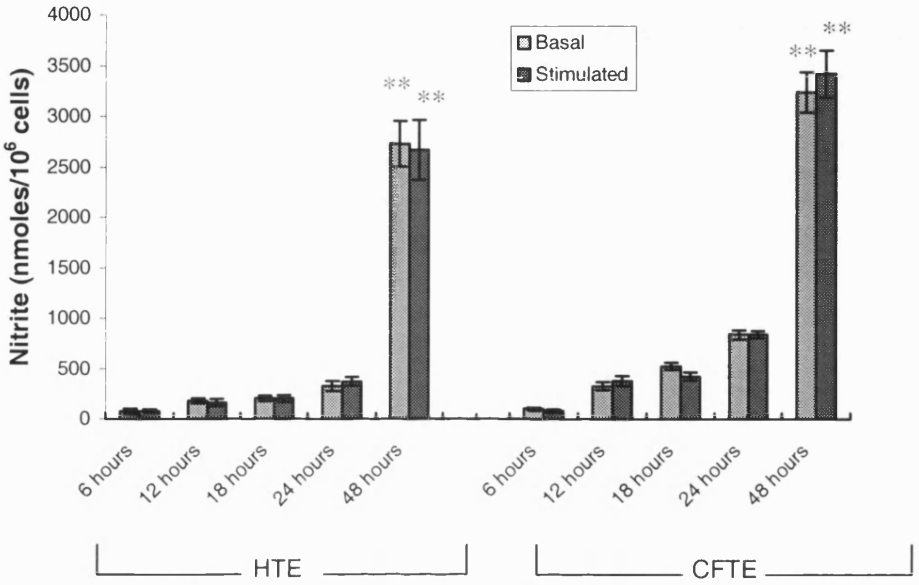


Figure 14 Time course of nitrite production by 9HTEo$^-$ and ΣCFTE29o$^-$ cells after incubation at 37°C with vehicle or cytomix (IFNγ 100 units/ml, IL-1β 3 ng/ml and TNFα 30 ng/ml). The cells were serum starved for 12 hours prior to treatment. 1 mM L-arginine was added to each stimulation. Each bar is the mean ± SEM of three experiments. (** $p<0.01$, compared with other time points).

To examine the possibility of substrate competition as a limiting factor for iNOS activity, the effect of cytomix on nitrite generation by confluent monolayers of airway epithelial cells was examined after 30 minutes pre-treatment with 20 mM of the arginase inhibitor L-norvaline. Arginase has been shown to have a high activity in activated in macrophages, where it metabolises L-arginine to form ornithine and urea (Granger *et al.*, 1990; Hrabak *et al.*, 1994). As arginase and NOS both use L-arginine as a substrate, it is possible that NO production by iNOS in airway epithelial cells may be regulated by arginase through competition for intracellular L-arginine. However, arginase inhibition had no significant effect on the levels of nitrite produced by 9HTEo$^-$ and ΣCFTE29o$^-$ cells after cytomix stimulation compared to the constitutive nitrite produced by vehicle treated cells (Fig 15).

Recently, it has been shown that iNOS gene expression in human airway epithelial cells occurs in response to cytokines in combination with double-stranded RNA (dsRNA) (Uetani *et al.*, 2001). In light of these findings, dsRNA was included in a series of 24-hour cytokine stimulations with the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines. Fig 16 reveals that stimulation of either cell line with dsRNA alone (10-100 μg/ml) did not affect nitrite generation compared with basal levels. In contrast to Uetani *et al.* (2001) the combination of cytomix and dsRNA (10-100 μg/ml) also had no significant effect on the levels of nitrite produced. This was the case for both cell types.

To investigate further the production and regulation of nitric oxide by airway epithelial cells, 24-hour cytokine stimulations were carried out in the presence and absence of a range of inhibitors. Fig 17 shows the effect of the specific iNOS inhibitor 1400W (3-30 μM) on nitrite generation by 9HTEo$^-$ and ΣCFTE29o$^-$ cells. The inhibitor was

added to the cells at the same time as the cytokines. 1400W did not have any significant effect, at any concentration, on nitrite levels in either basal or cytokine stimulated conditions. However, the use of the more general NOS inhibitor, aminoguanidine (0.5-1 mM) did have an effect on the levels of nitrite produced by the airway epithelial cells. As with 1400W, this inhibitor was added to the stimulations at the same time as the cytokines. Aminoguanidine (0.5 mM) significantly reduced basal nitrite production by the 9HTEo$^-$ cell line from 332.68 ± 38.32 nmoles/10^6 cells to 153.95 ± 22.29 nmoles/10^6 cells ($p<0.01$) and had a similar effect on nitrite production by the ΣCFTE29o$^-$ cell line (807.77 ± 38.64 nmoles/10^6 cells to 504.00 ± 47.90 nmoles/10^6 cells, $p<0.01$) (Fig 18). Aminoguanidine (1 mM) also significantly reduced basal nitrite production by both cell lines ($p<0.01$). In the cytokine stimulated conditions both concentrations of aminoguanidine significantly reduced nitrite generation in both cell types ($p<0.05$). Finally, pre-treatment of the airway epithelial cells with the protein synthesis inhibitor cycloheximide (10-100 μM) for 30 minutes prior to stimulation with cytomix had no significant effect on nitrite production by either cell line. In addition, treatment of unstimulated cells with this inhibitor had no effect on basal nitrite production (Fig 19).

To examine the possible modulatory effect of the phosphatidylinositol 3-kinase inhibitor LY294002 and the T helper 2 (Th2) cytokine IL-13 on nitrite generation, confluent monolayers of airway epithelial cells were incubated with various concentrations of LY294002 (3-30 μM) and IL-13 (3-30 ng/ml) 30 minutes prior to stimulation with cytomix. LY294002 had no significant effect on nitrite production by either cell line. This was also the case when unstimulated cells were treated with this inhibitor (Fig 20).

Fig 21 shows that treatment of the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines with IL-13 also had no effect on nitrite generation in basal or stimulated conditions.

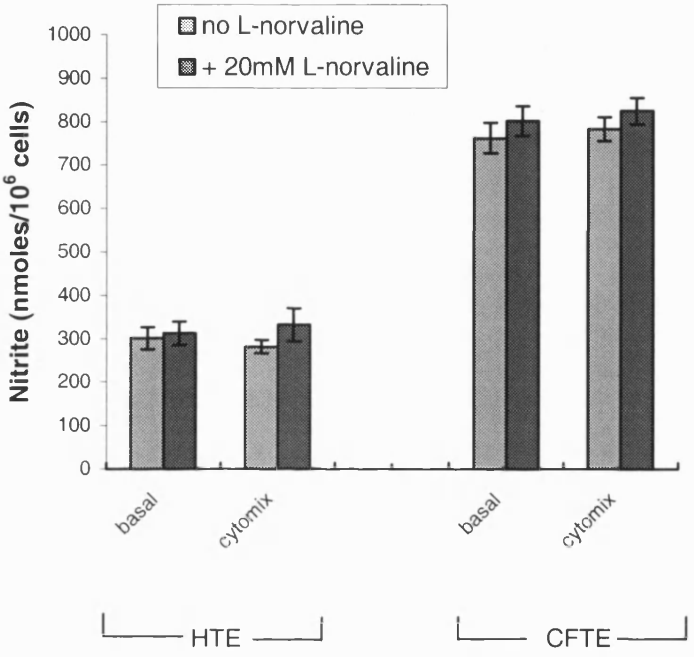


Figure 15 The effect of the arginase inhibitor L-norvaline (20 mM) on nitrite production by 9HTEo$^-$ and ΣCFTE29o$^-$ cells after 24 h incubation at 37°C with vehicle or cytomix (IFNγ 100 units/ml, IL-1β 3 ng/ml and TNFα 30 ng/ml). The cells were serum starved for 12 hours prior to treatment. L-norvaline was added 30 minutes before the cytokines. 1 mM L-arginine was added to each stimulation. Each bar is the mean ± SEM of three experiments.

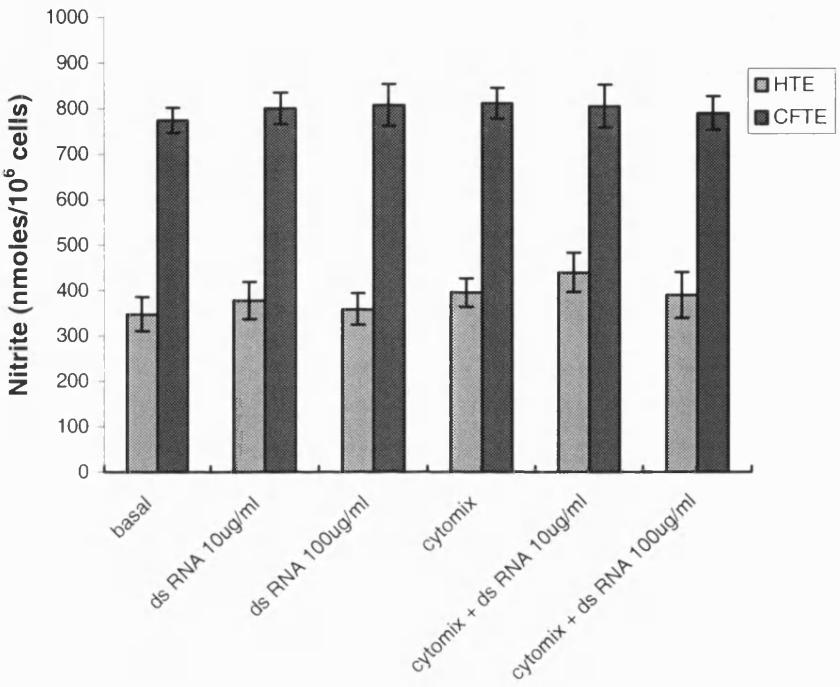


Figure 16 Nitrite production by 9HTEo$^-$ and ΣCFTE29o$^-$ cells after 24 h incubation at 37°C with vehicle, cytomix and double stranded RNA (10-100 μg/ml) added alone or in combination. The cells were serum starved for 12 hours prior to treatment. 1 mM L-arginine was added to each stimulation. Each bar is the mean ± SEM of three experiments.

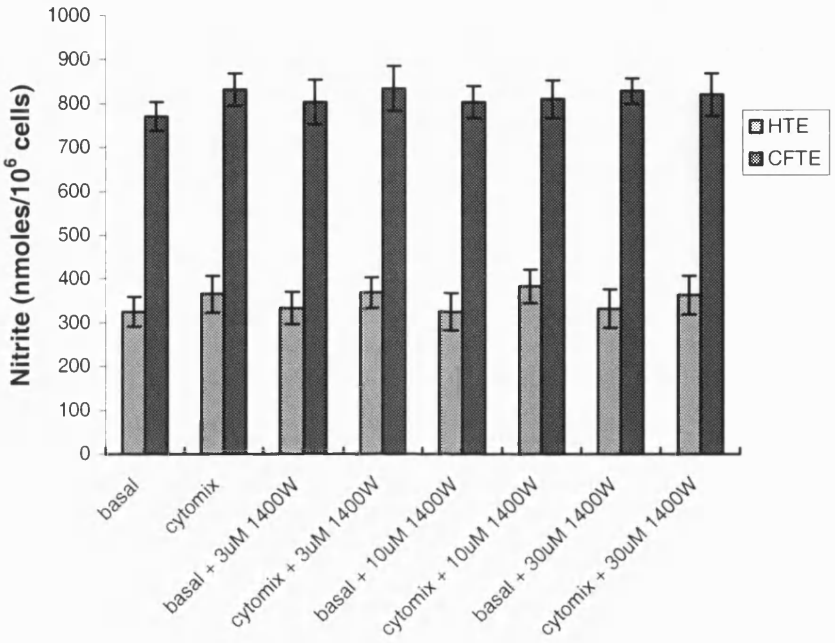


Figure 17 The effect of the iNOS inhibitor 1400W (3-30 μM) on nitrite production by 9HTEo⁻ and ΣCFTE29o⁻ cells after 24 h incubation at 37°C with vehicle or cytomix (IFNγ 100 units/ml, IL-1β 3 ng/ml, TNFα 30 ng/ml). The cells were serum starved for 12 hours prior to treatment. 1400W was added at the same time as the cytokines. 1 mM L-arginine was added to each stimulation. Each bar is the mean ± SEM of three experiments.

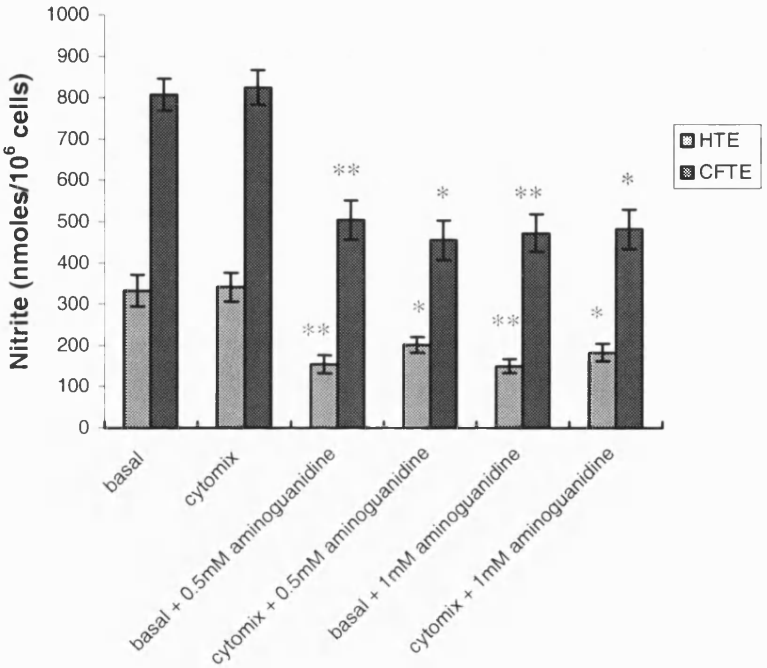


Figure 18 The effect of the general NOS inhibitor aminoguanidine (0.5-1 mM) on nitrite production by 9HTEo⁻ and ΣCFTE29o⁻ cells after 24 h incubation at 37°C with vehicle or cytomix (IFNγ 100 units/ml, IL-1β 3 ng/ml, TNFα 30 ng/ml). The cells were serum starved for 12 hours prior to treatment. Aminoguanidine was added at the same time as the cytokines. 1 mM L-arginine was added to each stimulation. Each bar is the mean ± SEM of three experiments (* $p<0.05$ compared with control, ** $p<0.01$ compared with control).

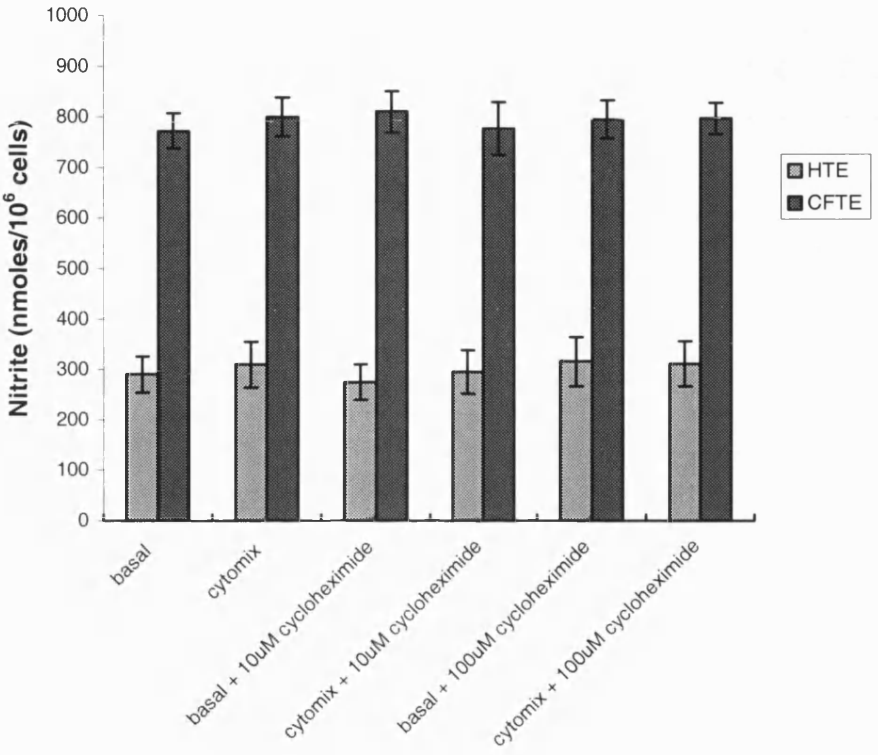


Figure 19 The effect of the protein synthesis inhibitor cycloheximide (10-100 μM) on nitrite production by 9HTEo⁻ and ΣCFTE29o⁻ cells after 24 h incubation at 37°C with vehicle or cytomix (IFNγ 100 units/ml, IL-1β 3 ng/ml, TNFα 30 ng/ml). The cells were serum starved for 12 hours prior to treatment. Cycloheximide was added 30 minutes before the cytokines. 1 mM L-arginine was added to each stimulation. Each bar is the mean ± SEM of three experiments.

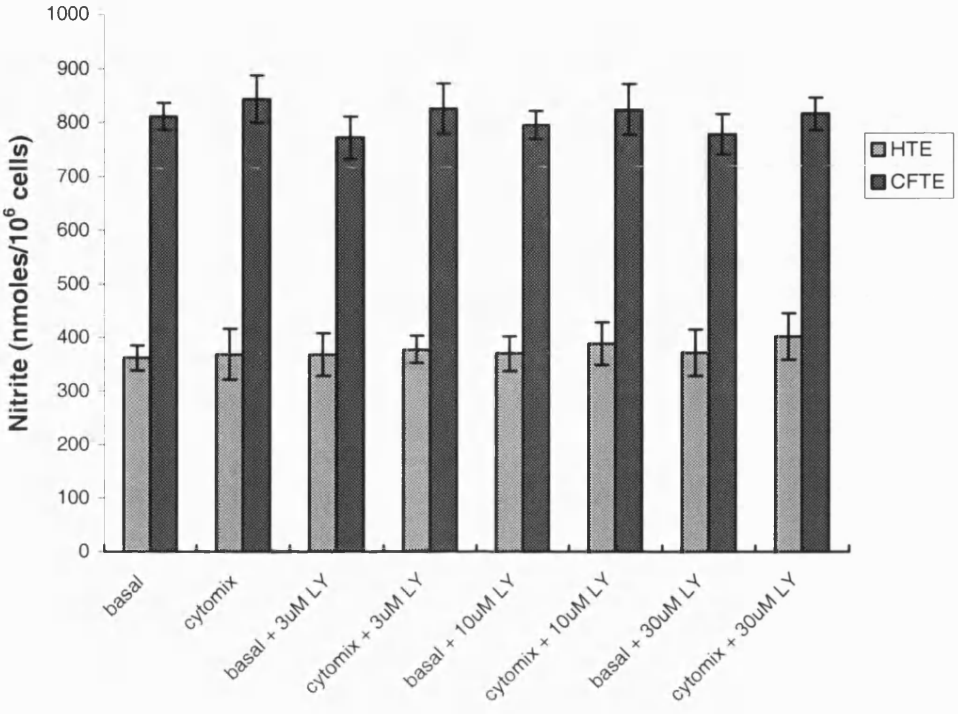


Figure 20 The effect of the phosphatidylinositol 3-kinase inhibitor LY294002 (3-30 μM) on nitrite production by 9HTEo$^-$ and ΣCFTE29o$^-$ cells after 24 h incubation at 37°C with vehicle or cytomix (IFNγ 100 units/ml, IL-1β 3 ng/ml, TNFα 30 ng/ml). The cells were serum starved for 12 hours prior to treatment. LY294002 was added 30 minutes before the cytokines. 1 mM L-arginine was added to each stimulation. Each bar is the mean ± SEM of three experiments.

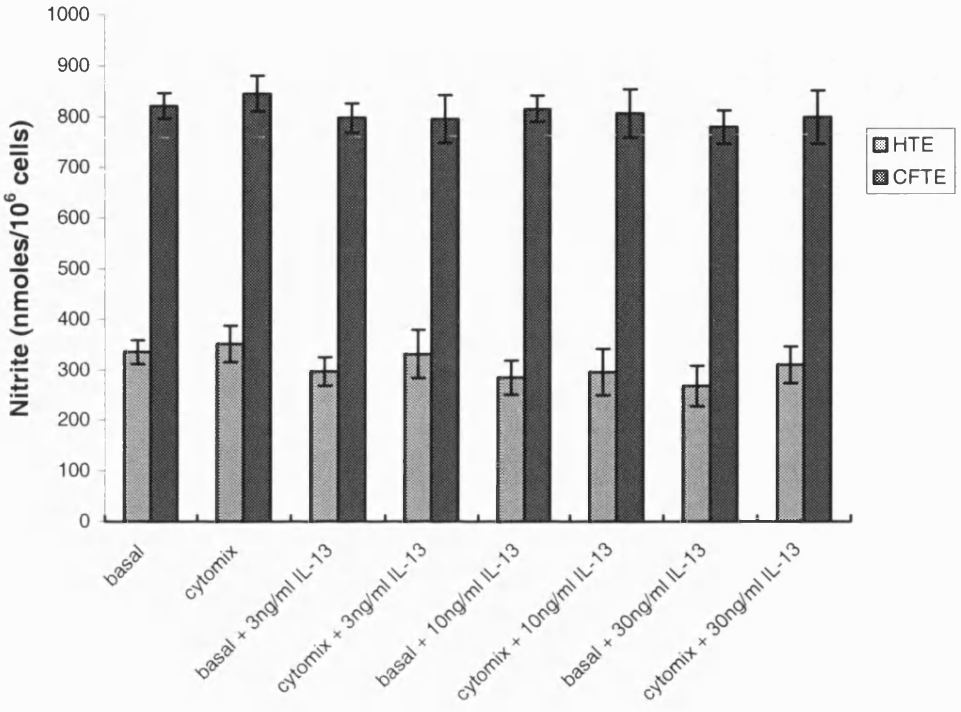


Figure 21 The effect of IL-13 (3-30 ng/ml) on nitrite production by 9HTEo$^-$ and ΣCFTE29o$^-$ cells after 24 h incubation at 37°C with vehicle or cytomix (IFNγ 100 units/ml, IL-1β 3 ng/ml, TNFα 30 ng/ml). The cells were serum starved for 12 hours prior to treatment. IL-13 was added 30 minutes before the cytokines. 1 mM L-arginine was added to each stimulation. Each bar is the mean ± SEM of three experiments.

3.4 NITRITE GENERATION BY pCEP9HTEo⁻/#2 AND pCEP9HTEo⁻/RF CELLS

As with the 9HTEo⁻ and ΣCFTE29o⁻ cell lines both pCEP lines produced a constitutive amount of nitrite in the absence of cytokine stimulation (351.87 ± 43.32 nmoles/10^6 cells for pCEP9HTEo⁻/#2 cells and 606.80 ± 45.93 nmoles/10^6 cells for pCEP9HTEo⁻/RF cells). Although the constitutive level of nitrite produced by the pCEP9HTEo⁻/RF cell line was less than that produced by the ΣCFTE29o⁻ cell line, it was still significantly higher than that produced by the pCEP9HTEo⁻/#2 cells ($p<0.05$) (Fig 22a). Again, as with the 9HTEo⁻ and ΣCFTE29o⁻ cell lines, increasing concentrations of the proinflammatory cytokines IFNγ, IL-1β and TNFα, added alone to either cell line, had no effect on nitrite generation compared to the constitutive nitrite production by vehicle treated cells (Figs 22 & 23). Similarly, combinations of IFNγ/IL-1β and IFNγ/TNFα had no effect on the amount of nitrite produced (Figs 24 & 25). Stimulation of the cells with high concentrations of the three proinflammatory cytokines combined also had no effect on the amount of nitrite generated compared to constitutive levels (Fig 26).

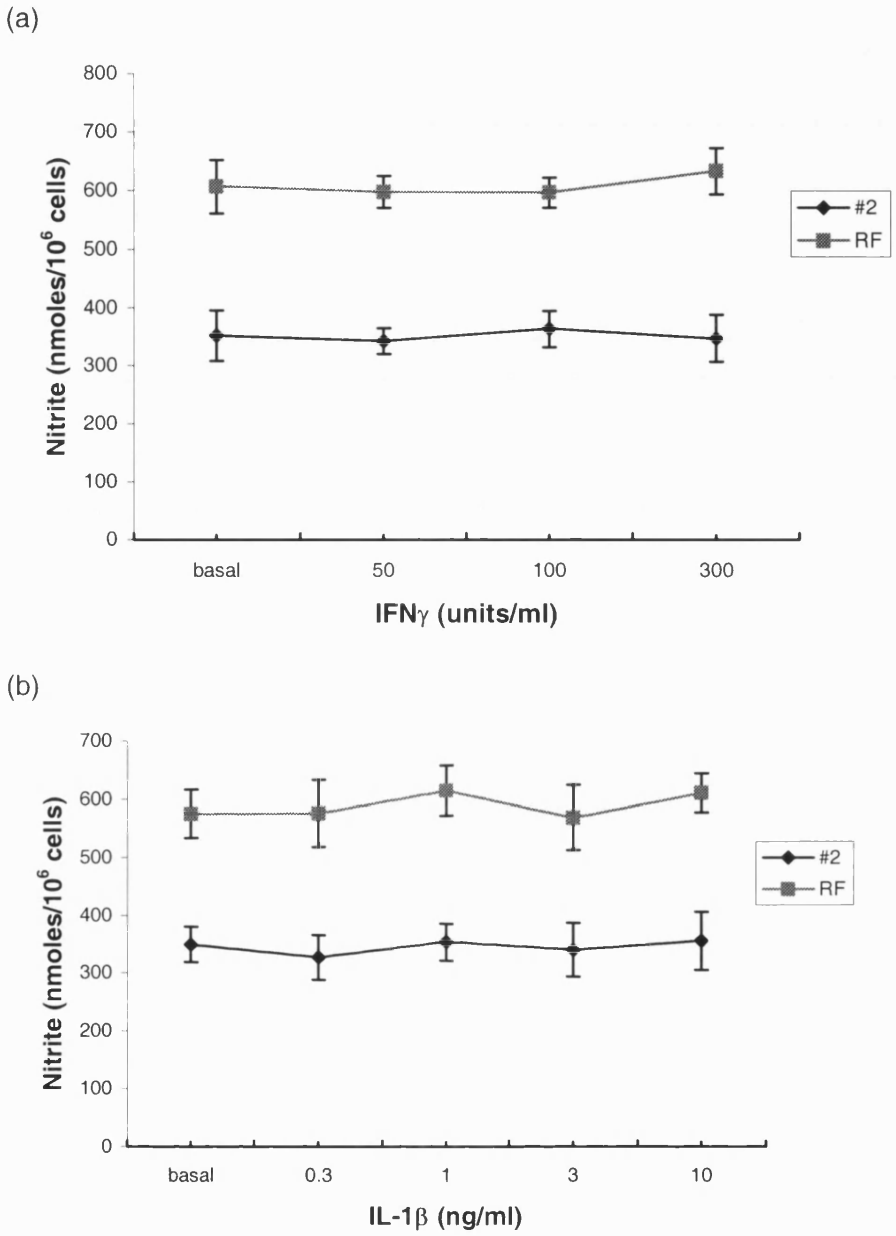


Figure 22 Nitrite production by 9HTEo⁻/pCEP#2 and 9HTEo⁻/pCEPRF cells after 24 h treatment at 37°C with (a) increasing concentrations of IFNγ (50-300 units/ml) and (b) increasing concentrations of IL-1β (0.3-10 ng/ml). The cells were serum starved for 12 hours prior to treatment. 1 mM L-arginine was added to each stimulation. Basal is the amount of nitrite produced in the presence of vehicle alone. Each point is the mean ± SEM of three experiments.

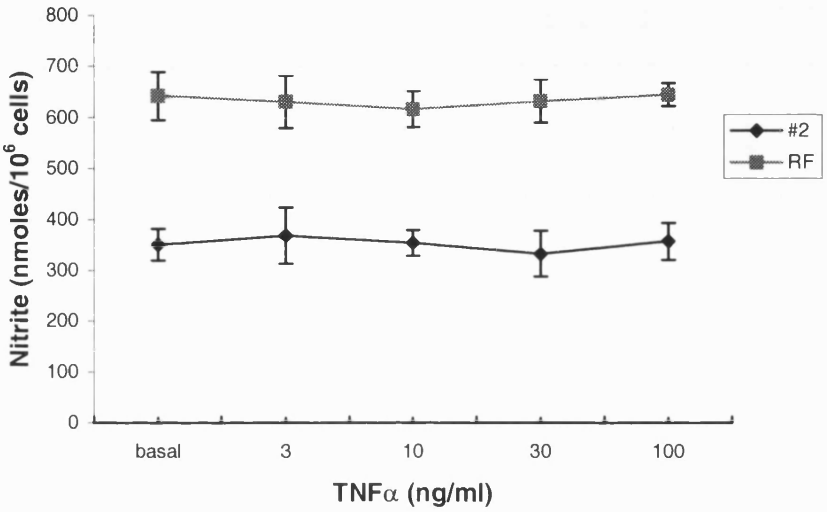


Figure 23 Nitrite production by 9HTEo⁻/pCEP#2 and 9HTEo⁻/pCEPRF cells after 24 h treatment at 37°C with increasing concentrations of TNFα (3-100 ng/ml). The cells were serum starved for 12 hours prior to treatment. 1 mM L-arginine was added to each stimulation. Basal is the amount of nitrite produced in the presence of vehicle alone. Each point is the mean ± SEM of three experiments.

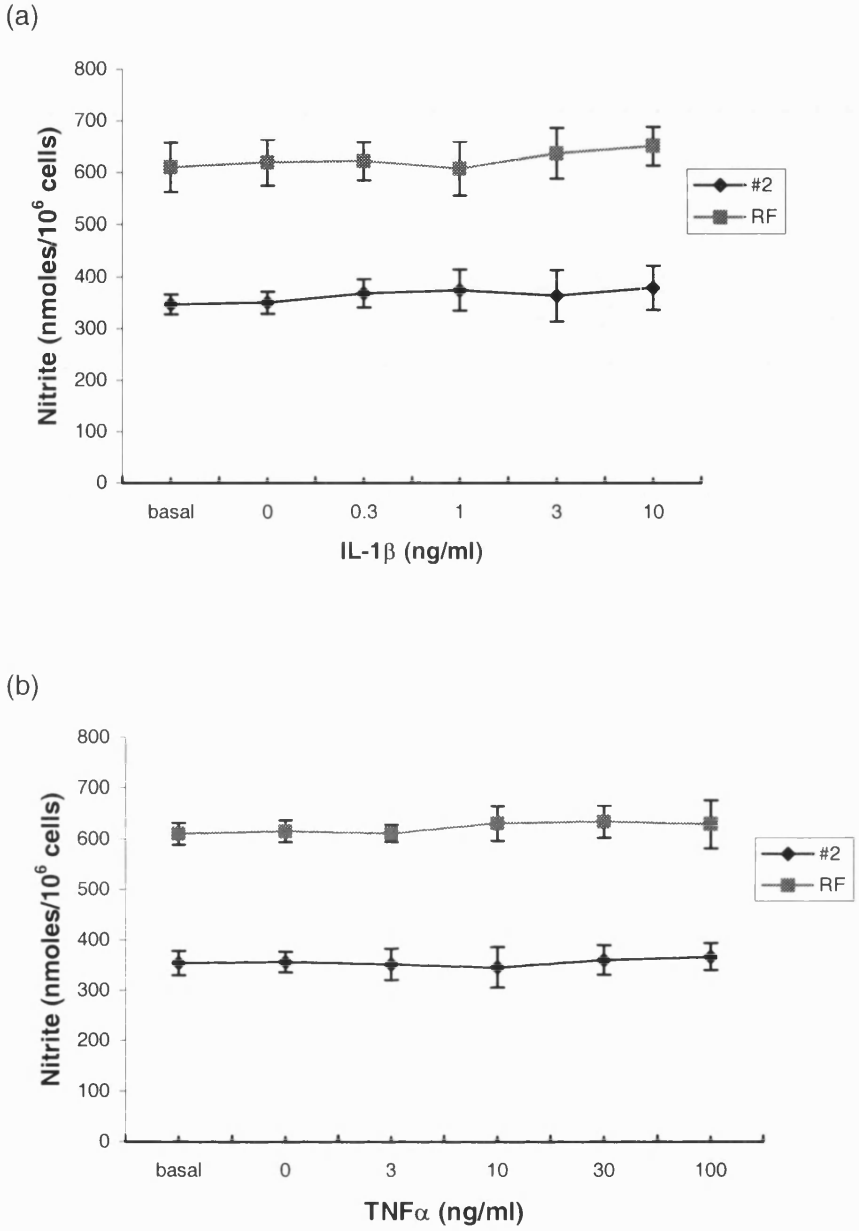


Figure 24 Nitrite production by 9HTEo⁻/pCEP#2 and 9HTEo⁻/pCEPRF cells after 24 h treatment at 37°C with (a) increasing concentrations of IL-1β (0-10 ng/ml) and (b) increasing concentrations of TNFα (0-100 ng/ml) in the presence of IFNγ 100 units/ml. The cells were serum starved for 12 hours prior to treatment. 1 mM L-arginine was added to each stimulation. Basal is the amount of nitrite produced in the presence of vehicle alone. Each point is the mean ± SEM of three experiments.

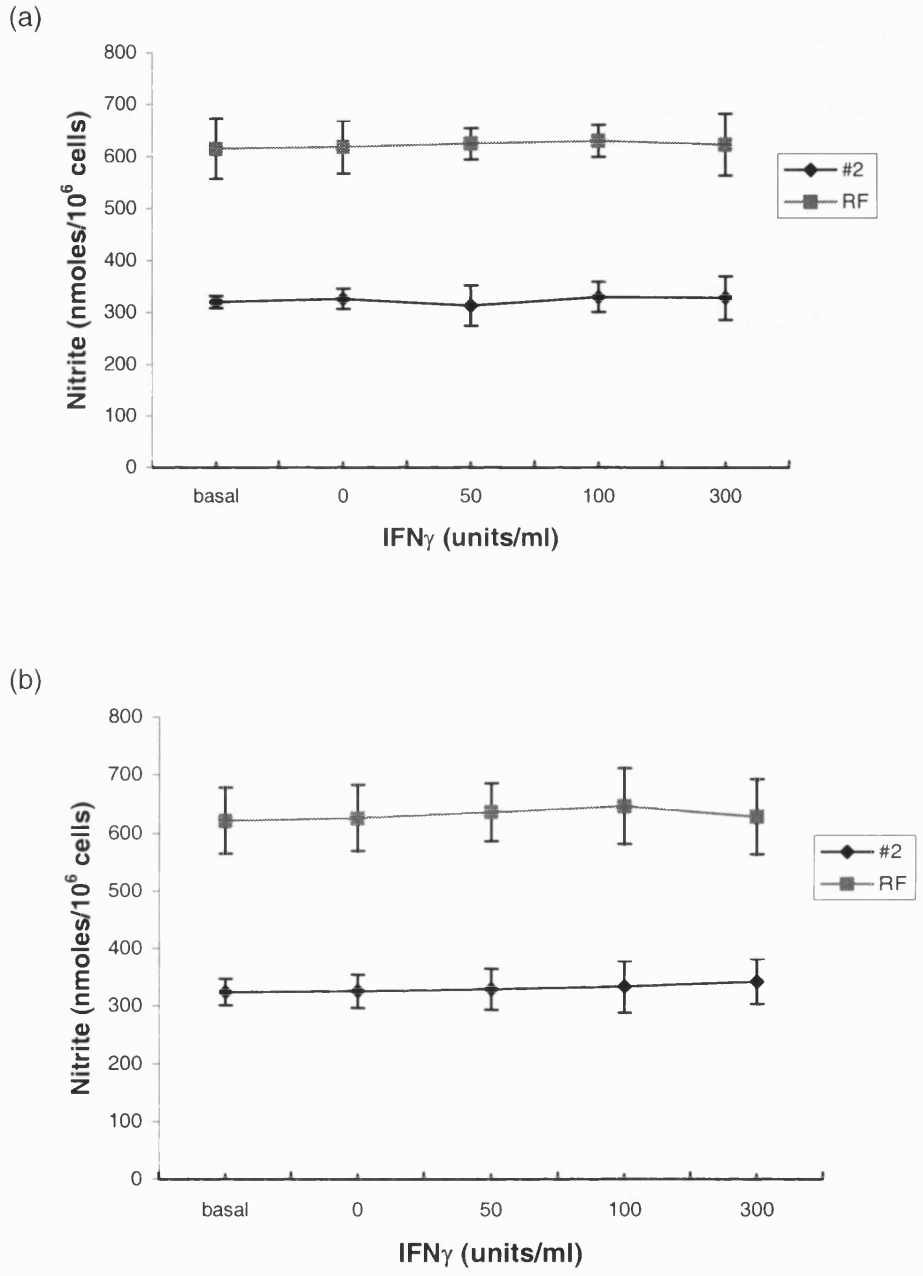


Figure 25 Nitrite production by 9HTEo⁻/pCEP#2 and 9HTEo⁻/pCEPRF cells after 24 h treatment at 37°C with increasing concentrations of IFNγ (0-300 units/ml) in the presence of (a) IL-1β (3 ng/ml) and (b) TNFα (30 ng/ml). The cells were serum starved for 12 hours prior to treatment. 1 mM L-arginine was added to each stimulation. Basal is the amount of nitrite produced in the presence of vehicle alone. Each point is the mean ± SEM of three experiments.

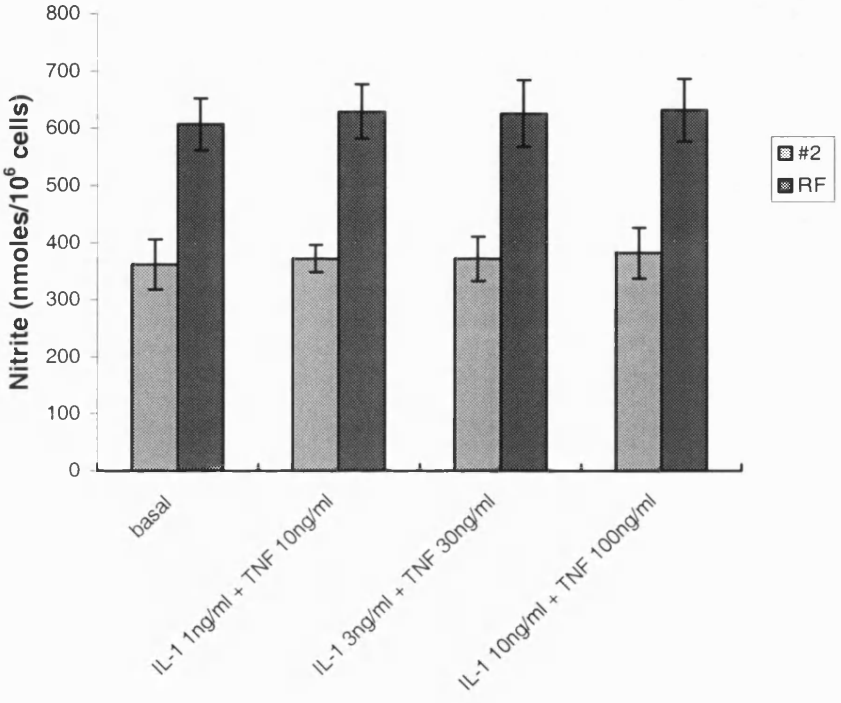


Figure 26 Nitrite production by 9HTEo⁻/pCEP#2 and 9HTEo⁻/pCEPRF cells after 24 h incubation at 37°C with IFNγ (100 units/ml), IL-1β (1-10 ng/ml) and TNFα (10-100 ng/ml) added in combination. The cells were serum starved for 12 hours prior to treatment. 1 mM L-arginine was added to each stimulation. Basal is the amount of nitrite produced in the presence of vehicle alone. Each bar is the mean ± SEM of three experiments.

As previously, time course studies were carried out to investigate if stimulation of the airway epithelial cells with cytomix (IFNγ 100 units/ml, IL-1β 3 ng/ml and TNFα 30 ng/ml) resulted in nitric oxide induction at timepoints other than 24 hours. Once again a time-dependent increase in constitutive nitrite levels was revealed for both cell lines (Fig 27). At each timepoint other than 48 h, the level of nitrite produced by the 9HTEo$^-$ /pCEPRF cell line was significantly greater than that produced by the 9HTEo$^-$/pCEP#2 cell line ($p<0.05$). Stimulation of either cell line with cytomix did not significantly increase the levels of nitrite produced at any time point. As observed with the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines, the amount of nitrite in the cell culture supernatant of both pCEP lines at 48 h was significantly larger than that produced at other time points ($p<0.01$) in both basal and stimulated conditions. Cell viability at this time point, examined by trypan blue exclusion, was found to be 62% for 9HTEo$^-$/pCEP#2 cells and 58% for 9HTEo$^-$/pCEPRF cells. At all other time points cell viability was always greater than 90%.

Table 1 summarises the production of nitrite by 9HTEo$^-$/pCEP#2 and 9HTEo$^-$/pCEPRF cells after 24h treatment at 37°C with a variety of cytokine and inhibitor combinations. Pre-treatment of the pCEP cell lines for 30 minutes with 20 mM of the arginase inhibitor L-norvaline had no significant effect on the levels of nitrite produced by the cells after cytomix stimulation compared to the levels of nitrite produced in the absence of the inhibitor. In addition, the combination of cytomix and dsRNA (100 μg/ml) had no significant effect on the levels of nitrite produced by the pCEP cells. The inclusion of the specific iNOS inhibitor 1400W (10 μM) with cytomix had no significant effect on nitrite levels in either cell line. However, as with the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines,

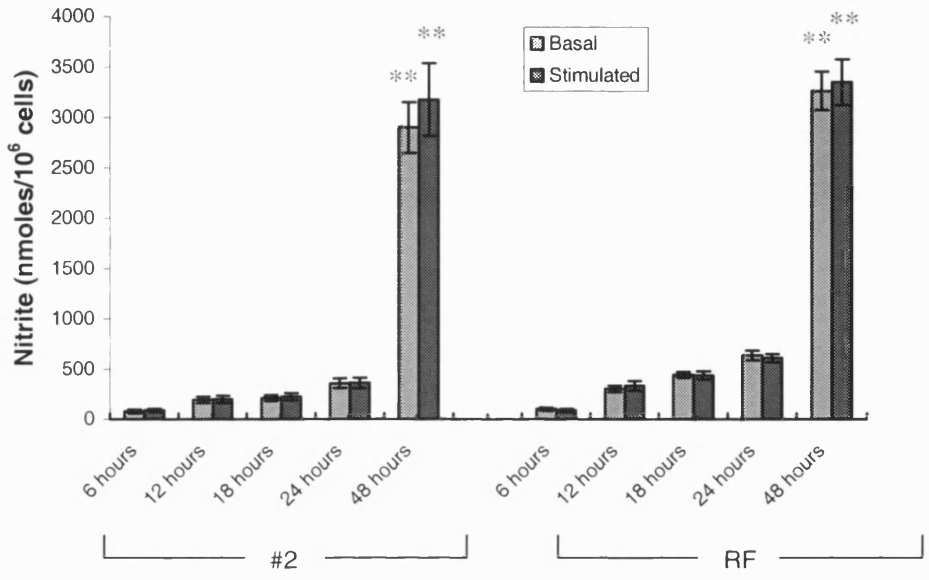


Figure 27 Time course of nitrite production by 9HTEo⁻/pCEP#2 and 9HTEo⁻ /pCEPRF cells after incubation at 37°C with vehicle or cytomix (IFNγ 100 units/ml, IL-1β 3 ng/ml and TNFα 30 ng/ml). The cells were serum starved for 12 hours prior to treatment. 1 mM L-arginine was added to each stimulation. Each bar is the mean ± SEM of three experiments (** $p<0.01$ compared with other time points).

aminoguanidine (0.5 mM) did have an effect on nitrite generation, significantly reducing nitrite production in cytokine stimulated pCEP cells from 367.53 ± 35.93 nmoles/10^6 cells to 199.49 ± 34.46 nmoles/10^6 cells for the 9HTEo$^-$/pCEP#2 cell line and from 611.05 ± 56.77 nmoles/10^6 cells to 356.84 ± 22.44 nmoles/10^6 cells for the 9HTEo$^-$ /pCEPRF cell line ($p<0.05$). Pre-incubation of the airway epithelial cells with cycloheximide (10 μM) for 30 minutes prior to stimulation with cytomix had no significant effect on nitrite production by either cell line. Finally, neither 30-minute pre-treatment with LY294002 (10 μM) nor IL-13 (30 ng/ml) had any significant effect on nitrite production by either pCEP cell line.

9HTEo⁻/pCEP#2

Treatment	Mean nmoles nitrite/10^6 cells
basal	352.43 ± 32.20
cytomix	367.53 ± 35.93
cytomix + 20 mM L-norvaline	346.55 ± 35.64
IL-1β 3 ng/ml + TNFα 30 ng/ml + dsRNA 100 μg/ml	342.94 ± 29.79
cytomix + 10 μM 1400W	360.69 ± 60.53
cytomix + 0.5 mM aminoguanidine	199.49 ± 34.46 *
cytomix + 10 μM cycloheximide	382.84 ± 37.30
cytomix + 10 μM LY294002	334.81 ± 21.95
cytomix + 30 ng/ml IL-13	348.05 ± 36.01

9HTEo⁻/pCEPRF

Treatment	Mean nmoles nitrite/10^6 cells
basal	629.64 ± 42.04
cytomix	611.05 ± 56.77
cytomix + 20 mM L-norvaline	624.04 ± 44.60
IL-1β 3 ng/ml + TNFα 30 ng/ml + dsRNA 100 μg/ml	613.04 ± 46.16
cytomix + 10 μM 1400W	590.35 ± 47.75
cytomix + 0.5 mM aminoguanidine	356.84 ± 22.44 *
cytomix + 10 μM cycloheximide	649.49 ± 32.27
cytomix + 10 μM LY294002	613.47 ± 19.97
cytomix + 30 ng/ml IL-13	638.02 ± 44.41

Table 1 Summary of nitrite production by 9HTEo⁻/pCEP#2 and 9HTEo⁻/pCEPRF cells after 24 h treatment at 37°C with a variety of cytokine and inhibitor combinations. The cells were serum starved for 12 hours prior to treatment. 1 mM L-arginine was added to each stimulation. Cytomix: IFNγ 100 units/ml, IL-1β 3 ng/ml, TNFα 30 ng/ml. Each value is the mean ± SEM of three experiments (* $p<0.05$ compared with control group).

3.5 DISCUSSION

Pulmonary disease in CF is characterised by long-lasting bacterial infection and chronic inflammation. Since high levels of NO have been shown to correlate with inflamed airways (Kharitonov *et al.*, 1994; Lundberg *et al.*, 1996), it might be expected that exhaled air from the lungs of CF patients should contain high levels of NO. However, it has been suggested that exhaled NO levels are reduced in CF patients compared with normal subjects and patients with other inflammatory airway diseases (Light *et al.*, 1989; Grasemann *et al.*, 1997). The observation by Kelley & Drumm (1998) that iNOS expression is reduced in airway epithelium from CF mice provides support for these findings and offers an explanation for the persistence of bacterial infections in the CF lung. However, other studies examining iNOS expression in human airway epithelial cells have conflicted with these findings. For example, the discovery that primary airway epithelial cells from humans rapidly lose their ability to synthesise iNOS mRNA *ex vivo* (Guo *et al.*, 1995) and the fact that some immortalised human airway epithelial cells show increased iNOS expression in response to proinflammatory cytokine stimulation (Asano *et al.*, 1994; Robbins *et al.*, 1994; Guo *et al.*, 1997; Watkins *et al.*, 1997) whereas others are unresponsive (Guo *et al.*, 1997), have made it difficult to accurately study NO biology in human airway epithelium. In the light of these findings, it seemed reasonable to investigate the production and regulation of NO by human airway epithelial cells and to identify potential differences in CF versus normal cells.

Each cell line studied produced a constitutive amount of nitrite in the absence of cytokine stimulation. The 9HTEo$^-$ and 9HTEo$^-$/pCEP#2 cell lines produced basal levels of nitrite similar to those produced by unstimulated BEAS-2B cells (an SV-40

immortalised human bronchial epithelial cell line) (Watkins *et al.*, 1997). Interestingly, the ΣCFTE29o$^-$ and 9HTEo$^-$/pCEPRF cell lines produced significantly more basal nitrite than their normal phenotype counterparts ($p<0.01$ for 9HTEo$^-$ / ΣCFTE29o$^-$ cells and $p<0.05$ for 9HTEo$^-$/pCEP#2 / 9HTEo$^-$/pCEPRF cells). At first glance these data would seem to refute the findings of Kelley & Drumm (1998) and those of Meng *et al.* (1998), who stated that iNOS expression (and therefore presumably NO production) is reduced in the human CF bronchial epithelial cell line CFBE41o$^-$. However, these nitrite levels are on a par with those produced by unstimulated A549 cells (a non-CF, type II alveolar carcinoma cell line) (Asano *et al.*, 1994; Robbins *et al.*, 1994) and are much less than the levels of nitrite produced by unstimulated primary airway epithelial cells from normal patients (Donnelly & Barnes, 2002). It is therefore difficult to ascertain whether or not the differences in basal nitrite between the CF and non-CF cell lines have any relevance. This is particularly true for the 9HTEo$^-$ and ΣCFTE29o$^-$ cells, as they are not a genetically matched pair of cell lines. However, the 9HTEo$^-$/pCEP#2 and 9HTEo$^-$ /pCEPRF cell lines are genetically matched and any differences observed between them are potentially more valid. It is possible that the higher levels of basal nitrite produced by the 9HTEo$^-$/pCEPRF cell line are a consequence of the cells having repressed function, although variability between clones cannot be excluded.

Stimulation of the cell lines over 24 hours with IFNγ, IL-1β or TNFα alone had no effect on basal nitrite generation. This was to be expected, as although IFNγ alone has been shown to induce a high level of iNOS expression in primary cultured human airway epithelial cells (Guo *et al.*, 1997), no single cytokine has been found to increase iNOS expression in human airway epithelial cell lines. In fact, the proinflammatory cytokine requirement for the induction of iNOS in immortalised airway epithelial cells appears to

vary depending upon the cell line being studied. For example, the combination of IFNγ, IL-1β and TNFα leads to iNOS induction in A549 cells (Asano *et al.*, 1994; Robbins *et al.*, 1994; Guo *et al.*, 1997), whereas IFNγ, TNFα and LPS are required for iNOS induction in the BEAS-2B bronchial cell line (Watkins *et al.*, 1997). In contrast, the bronchial epithelial cell line BET-1A does not show any iNOS induction in response to proinflammatory cytokine combinations (Guo *et al.*, 1997). In this series of experiments, a variety of combinations of IFNγ, IL-1β and TNFα were tested on the 9HTEo$^-$, ΣCFTE29o$^-$, 9HTEo$^-$/pCEP#2 and 9HTEo$^-$/pCEPRF cell lines, but were found to have no effect on the amount of nitrite produced over a 24 hour period. Furthermore, the data from the time course studies revealed that these cytokines did not have any effect on nitrite generation by these cell lines at any time point from 6-48 hours.

The lack of response of the 9HTEo$^-$ / ΣCFTE29o$^-$ and 9HTEo$^-$/pCEP#2 / 9HTEo$^-$ /pCEPRF cell lines to cytokine stimulation was unlikely to be due to substrate limitation, as all the experiments were carried out in the presence of 1 mM L-arginine. This concentration of L-arginine has been shown to significantly increase nitrite output from cytokine stimulated BEAS-2B cells, without having any effect on unstimulated cells (Watkins *et al.*, 1997). In addition, the use of the specific arginase inhibitor L-norvaline removed the possibility of any competition for intracellular L-arginine between iNOS and arginase in these cell lines. L-norvaline has been shown to significantly increase NO production from LPS activated mouse macrophages (Chang *et al.*, 1998), with the effect being dose dependent up to 10 mM L-norvaline. Concentrations greater than 10 mM had no further effect on NO production. This suggests that the concentration of L-norvaline chosen for this series of experiments (20 mM) should have successfully inhibited any arginase present in the cells. The fact that L-norvaline had no effect on

cytokine stimulated nitrite generation in this study does not necessarily mean that arginase is absent from these airway epithelial cell lines. It is equally probable that some other factor or combination of factors were responsible for the lack of nitrite generation by the 9HTEo$^-$, ΣCFTE29o$^-$, 9HTEo$^-$/pCEP#2 and 9HTEo$^-$/pCEPRF cells in response to cytokine stimulation. If this were the case, it would conceal the effect of any arginase inhibition.

It is interesting to note from the time course data the magnitude of the nitrite levels produced by the cells upon 48-hour incubation with vehicle or cytomix. In the case of the 9HTEo$^-$ and 9HTEo$^-$/pCEP#2 cell lines, the levels of nitrite produced by unstimulated cells at 48 hours were 8 fold higher than the levels produced at 24 hours. For unstimulated ΣCFTE29o$^-$ cells the nitrite levels were 4 fold higher at 48 hours than at 24 hours and for unstimulated 9HTEo$^-$/pCEPRF cells the levels were 5 fold higher. A similar pattern was observed when the cells were stimulated with cytokines. These high nitrite levels correlated with an increase in cell debris and a decrease in cell number in each case. The most likely explanation for these findings is that the cells were cultured for too long in the absence of serum (they were serum starved for 12 hours prior to the experiment and then stimulated by vehicle or cytomix for 48 hours also in the absence of serum) and had started to die. It is possible that the dying cells released a large amount of NO, giving rise to the highly elevated nitrite readings.

The fact that different human airway epithelial cell lines show different cytokine requirements for the induction of iNOS suggests that the mechanisms regulating iNOS expression in human airway epithelial cells are complex and involve the activation of multiple signal transduction pathways. Recently, Uetani *et al.* (2001), have shown that

iNOS gene expression in BEAS-2B cells occurs in response to double-stranded RNA (dsRNA), through the rapid activation of dsRNA-activated protein kinase (PKR) and subsequent activation of signalling proteins including NF-κB and IRF-1. They have also verified a central role for dsRNA-activated pathways in generalised iNOS signalling, by demonstrating a loss of iNOS induction in response to a wide variety of cytokines and mediators in PKR-null cells (Uetani *et al.*, 2000). Uetani and colleagues (2001) did not show any iNOS induction in BEAS-2B cells in response to stimulation with IFNγ, TNFα and IL-1β combined. Nor did they show any change in iNOS expression when cells were incubated with dsRNA alone. It was only when dsRNA and proinflammatory cytokines were used together that iNOS mRNA could be detected in BEAS-2B cell lysates. This data appears to be in conflict with that of Watkins *et al.* (1997), who claim that iNOS mRNA is expressed in response to IFNγ, TNFα and LPS in BEAS-2B cells. However, Watkins and colleagues (1997) also state that the expression of iNOS mRNA in response to cytokine stimulation diminished rapidly with successive passages. It may well be that the BEAS-2B cells used by Uetani *et al.* (2001), were of a later passage than those used by Watkins *et al.* (1997). Uetani and colleagues (2001) claim that dsRNA is a physiologically relevant activator of gene expression in airway epithelial cells. The airway is frequently the first site of contact for viral infections and most viruses induce the synthesis of dsRNA at some time during their replication cycles (Jacobs & Langland, 1996; Maggi *et al.*, 2000). The involvement of dsRNA in the generation of NO by the airway epithelium may therefore be important in antiviral host defence.

In addition to their work with the BEAS-2B cell line, Uetani *et al.* (2001) also examined iNOS expression in BET-1A cells. Firstly, they established that these cells did not

express iNOS in response to proinflammatory cytokine stimulation. This finding supports the earlier work of Guo *et al.* (1997), who also examined iNOS expression in this cell line. As with the BEAS-2B cells, stimulation of BET-1A cells with dsRNA alone had no effect on iNOS induction. However, in contrast to the results obtained with the BEAS-2B cell line, incubation of BET-1A cells with a combination of cytokines and dsRNA also had no effect on iNOS expression. In conclusion, it appears that although both BEAS-2B and BET-1A cells are derived from normal bronchial epithelial cells (Reddel *et al.*, 1988), the conditions required for iNOS expression are different in the two cell lines. This may well be a result of how the cell lines were created. BEAS-2B cells were formed by transformation of primary human bronchial epithelial cells with adenovirus-12 SV40 hybrid virus defective in the SV40 origin of replication, whereas BET-1A cells were derived from primary human bronchial epithelial cells transformed using a retrovirus vector containing the SV40 large T antigen (Reddel *et al.*, 1988).

Stimulation of the 9HTEo$^-$ / ΣCFTE29o$^-$ and 9HTEo$^-$/pCEP#2 / 9HTEo$^-$/pCEPRF cell lines with proinflammatory cytokines combined with dsRNA had no effect on nitrite generation compared to basal levels. These cell lines are derived from tracheal epithelial cells and this could explain why they behave differently to the bronchial BEAS-2B cell line. However, there are also differences in the way these cell lines were created compared to both the BEAS-2B and BET-1A cell lines. Both the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines were created from primary human tracheal epithelial cells transformed using a plasmid containing an SV40 genome defective in the origin of replication (Gruenert *et al.*, 1988; Kunzelmann *et al.*, 1993). As discussed earlier, the pCEP cell lines are derived from 9HTEo$^-$ cells (Perez *et al.*, 1996) and have therefore undergone similar transformation procedures to this cell line. It is possible that

differences in transformation between cell lines and/or subsequent differences in genetic mutations are responsible for the observed variation in iNOS activity and subsequent nitrite production between the cell lines used in this study and those used elsewhere.

The cell lines used in this study did not produce nitrite above basal levels in response to any cytokine combination and it can therefore be assumed that no induction of iNOS occurred. In contrast, the combination of IFNγ, IL-1β and TNFα did lead to nitrite generation by the A549 alveolar cell line. This finding rules out the possibility that the cytokines were non-viable and provides support for the use of the fluorometric nitrite assay as a way of assessing iNOS induced NO production. Attempts were also made to examine iNOS induction by immunoblot and Northern blot analysis. However, no iNOS protein or iNOS message could be detected in any of the cell lysates (data not included). The inability to induce iNOS in these airway epithelial cell lines explains why the protein synthesis inhibitor, cycloheximide had little effect on the levels of nitrite generated. The basal levels of nitrite produced by these cell lines must have been due to the result of constitutive enzyme activity, which did not require *do novo* protein synthesis. The lack of iNOS induction also explains why the specific iNOS inhibitor, 1400W had no effect on cytokine stimulated nitrite levels. This inhibitor also failed to have any effect on unstimulated cells, making it very unlikely that the basal levels of nitrite produced by the cell lines were the result of constitutive iNOS activity. The more general NOS inhibitor, aminoguanidine did significantly decrease the levels of nitrite produced by the airway epithelial cells. This finding suggests that a large proportion of the nitrite generated in the absence of cytokine stimulation was the result of some kind of constitutive NOS activity. Constitutive NOS produce small amounts of NO (nM range) and this fits with the observed data.

The T helper 2 (Th2) cytokine interleukin-13 has been reported to inhibit NO production by activated murine peritoneal macrophages (Doyle *et al.*, 1994) and mouse bone marrow-derived macrophages (Doherty *et al.*, 1993). More recently Wright *et al.* (1997) demonstrated that IL-13 inhibits iNOS expression in the human colonic epithelial cell line HT-29 and the mechanism of this inhibition was linked to activation of the signalling enzyme phosphatidylinositol 3-kinase (PI3K). In support of this, IL-13 has been shown to inhibit NF-κB activation in some cell models (Manna & Aggarwai, 1998). To examine a possible link between PI3K and NO production in human airway epithelial cells, IL-13 and the PI3K inhibitor LY294002 were included in a series of 24-hour cytokine stimulations using the 4 cell lines under investigation. Unfortunately, it was not possible to demonstrate any links between IL-13, PI3K and NO production in these cells due to the inability to induce nitrite generation above basal levels.

3.6 SUMMARY OF RESULTS

- The 9HTEo$^-$, ΣCFTE29o$^-$, 9HTEo$^-$/pCEP#2 and 9HTEo$^-$/pCEPRF cell lines all produced nitrite in the absence of cytokine stimulation with the CF phenotype cells producing significantly more nitrite than the non-CF phenotype cells.
- Stimulation of the 9HTEo$^-$, ΣCFTE29o$^-$, 9HTEo$^-$/pCEP#2 and 9HTEo$^-$/pCEPRF cell lines with proinflammatory cytokines did not result in production of nitrite above basal levels at any time point from 6-48 hours.
- After 48 hours in the absence of serum, cell viability was considerably reduced for all four cell lines and this correlated with a high level of basal nitrite release.
- The use of the arginase inhibitor L-norvaline had no effect on the levels of nitrite produced in response to cytokine stimulation by the 9HTEo$^-$, ΣCFTE29o$^-$, 9HTEo$^-$ /pCEP#2 and 9HTEo$^-$/pCEPRF cell lines.
- The inclusion of dsRNA in the cytokine stimulations had no effect on the levels of nitrite produced by any of the four cell lines.
- Neither the specific iNOS inhibitor 1400W, nor the protein synthesis inhibitor cycloheximide, had any effect on the basal or cytokine stimulated nitrite levels produced by the four cell lines.
- The general NOS inhibitor aminoguanidine significantly reduced the basal levels of nitrite produced by all four cell lines.
- Neither LY294002 nor IL-13 had any effect on the levels of nitrite produced by the four cell lines.
- Stimulation of the type II alveolar carcinoma cell line A549 with IFNγ, IL-1β and TNFα resulted in a significant increase in nitrite production compared with basal levels.

4 INTERLEUKIN-8 PRODUCTION BY AIRWAY EPITHELIAL CELLS

4.1 INTRODUCTION

The aim of this work was to examine the mechanisms involved in the production and regulation of IL-8 by CF and non-CF human airway epithelial cells, using the 9HTEo$^-$ (non-CF), ΣCFTE29o$^-$ (CF), 9HTEo$^-$/pCEP#2 (empty vector transfected) and 9HTEo$^-$/pCEPRF (R domain overexpressing) cell lines as models of human airway epithelium. The supernatants assayed for IL-8 were from the same cell cultures as those assayed for nitrite levels, where confluent monolayers of cells were serum starved for 12 hours and subsequently stimulated for 24 hours at 37°C with vehicle or increasing concentrations of IFNγ (0-300 units/ml), IL-1β (0-10 ng/ml) and TNFα (0-100 ng/ml), either alone or in various combinations. IL-8 levels were determined in cell culture supernatants using an IL-8 enzyme-linked immunosorbant assay as described in the materials and methods section.

4.2 INTERLEUKIN-8 GENERATION BY 9HTEo$^-$ and ΣCFTE29o$^-$ CELLS

Both 9HTEo$^-$ and ΣCFTE29o$^-$ cells produced a small amount of IL-8 in the absence of cytokine stimulation (5.29 ± 0.91 ng/10^6 cells for 9HTEo$^-$ cells and 7.09 ± 0.90 ng/10^6 cells for ΣCFTE29o$^-$ cells) (Fig 28a). The differences between these levels was not found to be significant. Increasing concentrations of the proinflammatory cytokine IFNγ had no effect on basal IL-8 levels in either cell line (Fig 28a), whereas increasing concentrations of IL-1β and TNFα added alone did affect IL-8 generation (Figs 28b &

29). IL-8 production by the 9HTEo$^-$ cell line was significantly increased upon incubation with IL-1β 0.3 ng/ml from 5.41 ± 0.49 ng/10^6 cells under basal conditions to 15.14 ± 1.11 ng/10^6 cells after stimulation ($p<0.01$). Incubation of these cells with IL-1β 1 ng/ml further increased IL-8 generation to 24.08 ± 1.18 ng/10^6 cells. Higher concentrations of IL-1β (3 & 10 ng/ml) had no additional effects on IL-8 levels. In comparison, IL-8 production by the ΣCFTE29o$^-$ cell line significantly increased from 6.13 ± 0.36 ng/10^6 cells under basal conditions to 12.05 ± 1.01 ng/10^6 cells in the presence of IL-1β 3 ng/ml ($p<0.01$). Stimulation with IL-1β 10 ng/ml had no additional effect (Fig 28b). At all concentrations of IL-1β (0.3-10 ng/ml) 9HTEo$^-$ cells produced significantly more IL-8 than ΣCFTE29o$^-$ cells ($p<0.01$). In both cell lines, stimulation with low concentrations of TNFα (3 & 10 ng/ml) lead to reduced IL-8 production compared with vehicle treated cells (Fig 29). Stimulations involving higher concentrations of TNFα (30 & 100 ng/ml) significantly augmented IL-8 production by both cell lines ($p<0.01$). IL-8 generation increased to 17.00 ± 1.14 ng/10^6 cells for 9HTEo$^-$ cells stimulated with TNFα 100 ng/ml and to 10.85 ± 1.23 ng/10^6 cells for ΣCFTE29o$^-$ cells stimulated with this highest concentration of TNFα. In addition, at higher concentrations of TNFα (30 & 100 ng/ml) the 9HTEo$^-$ cell line produced significantly more IL-8 than the ΣCFTE29o$^-$ cell line ($p<0.01$).

The combination of IFNγ 100 units/ml and IL-1β (0-10 ng/ml) or TNFα (0-100 ng/ml) produced the same pattern of IL-8 generation as IL-1β or TNFα alone in both cell lines (Fig 30). Similarly, the combination of IL-1β (1-10 ng/ml) and TNFα (10-100 ng/ml) produced no significant differences in either cell line when compared with IL-1β alone (Fig 31). Stimulation of the cells with increasing concentrations of the three

proinflammatory cytokines combined did show an additive effect on IL-8 generation (Fig 32). Incubation of 9HTEo$^-$ cells with IFNγ 100 units/ml, IL-1β 1 ng/ml and TNFα 10 ng/ml produced significantly more IL-8 (34.21 ± 1.51 ng/10^6 cells) than stimulation with IL-1β 1 ng/ml and TNFα 10 ng/ml (26.92 ± 1.14 ng/10^6 cells) ($p<0.05$). Stimulation of this cell line with higher concentrations of the three cytokines combined also resulted in significantly increased IL-8 production compared to corresponding concentrations of IL-1β and TNFα in combination ($p<0.05$). A similar pattern was observed with the ΣCFTE29o$^-$ cell line. Incubation of ΣCFTE29o$^-$ cells with IFNγ 100 units/ml, IL-1β 1 ng/ml and TNFα 10 ng/ml lead to significantly increased IL-8 production (20.92 ± 1.46 ng/10^6 cells) compared to stimulation with IL-1β 1 ng/ml and TNFα 10 ng/ml (10.24 ± 0.91 ng/10^6 cells) ($p<0.05$). As with the 9HTEo$^-$ cell line, stimulation of ΣCFTE29o$^-$ cells with higher concentrations of the three cytokines combined also resulted in significantly increased IL-8 production compared to corresponding concentrations of IL-1β and TNFα in combination ($p<0.01$).

Time course studies were carried out to investigate if stimulation of the airway epithelial cells with IFNγ 100 units/ml, IL-1β 3 ng/ml and TNFα 30 ng/ml (cytomix) resulted in induction of IL-8 at any other timepoints. These studies revealed very little increase in basal IL-8 generation over time. However, a time-dependent increase in cytomix stimulated IL-8 production was observed for both cell lines over a 48 hour period (Fig. 33). At each timepoint the amount of cytokine stimulated IL-8 produced by both cell lines was signifcantly greater than the amount of IL-8 produced under basal

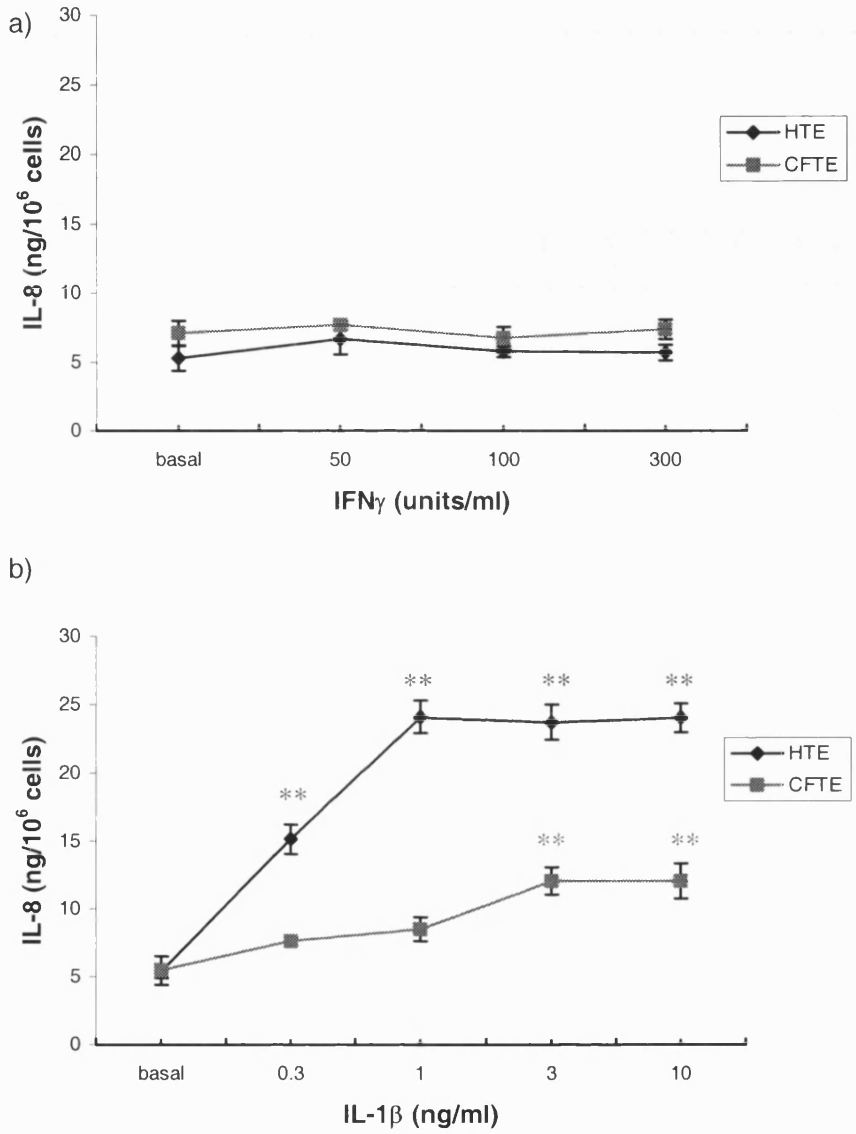


Figure 28 IL-8 production by 9HTEo$^-$ and ΣCFTE29o$^-$ cells after 24 h treatment at 37°C with (a) increasing concentrations of IFNγ (50-300 units/ml) and (b) increasing concentrations of IL-1β (0.3-10 ng/ml). The cells were serum starved for 12 hours prior to treatment. Basal is the amount of IL-8 produced in the presence of vehicle alone. Each point is the mean ± SEM of three experiments (** $p<0.01$ compared with basal).

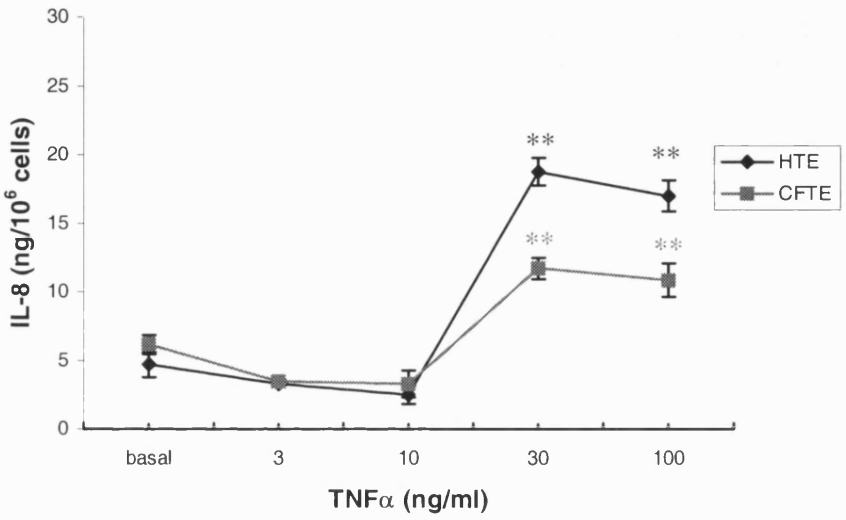


Figure 29 IL-8 production by 9HTEo$^-$ and ΣCFTE29o$^-$ cells after 24 h treatment at 37°C with increasing concentrations of TNFα (3-100 ng/ml). The cells were serum starved for 12 hours prior to treatment. Basal is the amount of IL-8 produced in the presence of vehicle alone. Each point is the mean ± SEM of three experiments (** $p<0.01$ compared with basal).

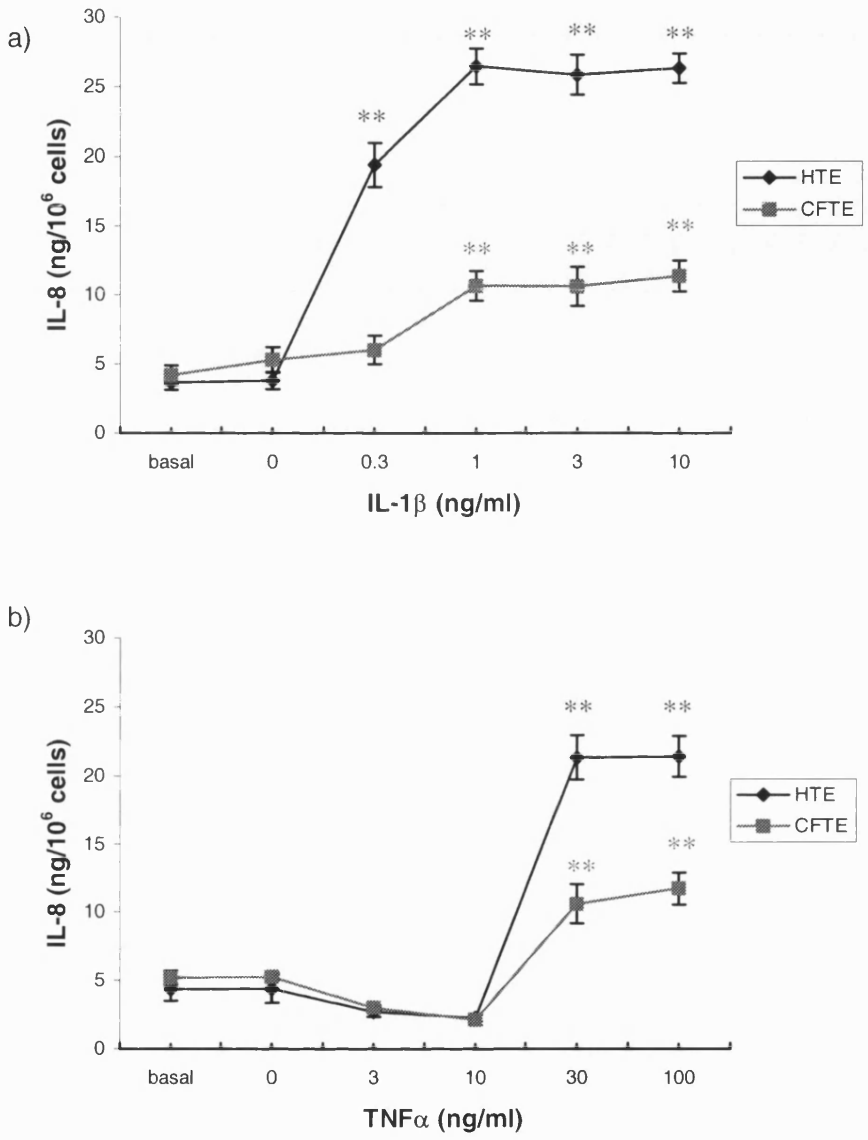


Figure 30 IL-8 production by 9HTEo$^-$ and ΣCFTE29o$^-$ cells after 24 h treatment at 37°C with (a) increasing concentrations of IL-1β (0-10 ng/ml) and (b) increasing concentrations of TNFα (0-100 ng/ml), in the presence of IFNγ 100 units/ml. The cells were serum starved for 12 hours prior to treatment. Basal is the amount of IL-8 produced in the presence of vehicle alone. Each point is the mean ± SEM of three experiments (** $p<0.01$ compared with basal).

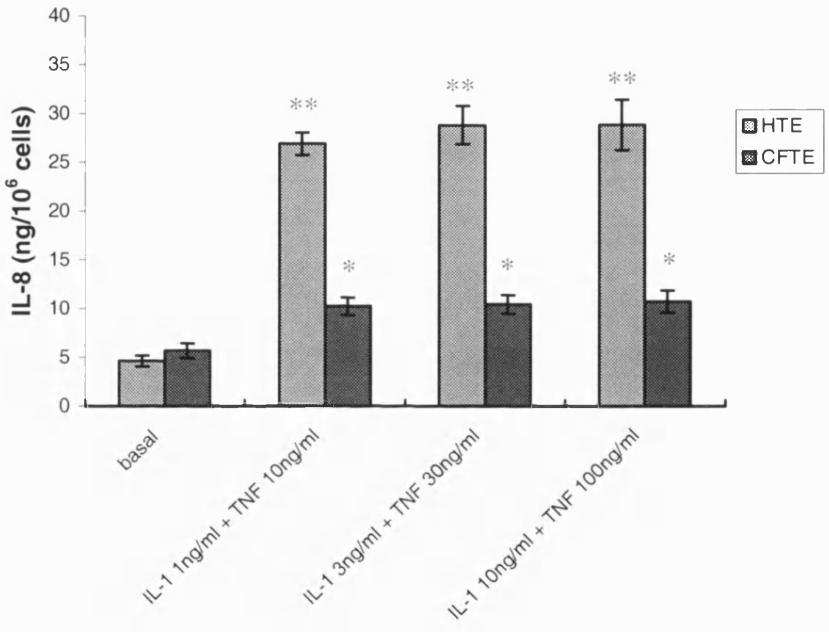


Figure 31 IL-8 production by 9HTEo$^-$ and ΣCFTE29o$^-$ cells after 24 h treatment at 37°C with IL-1β (1-10 ng/ml) and TNFα (10-100 ng/ml) added in combination. The cells were serum starved for 12 hours prior to treatment. Basal is the amount of IL-8 produced in the presence of vehicle alone. Each point is the mean ± SEM of three experiments (* $p<0.05$, ** $p<0.01$ compared with basal).

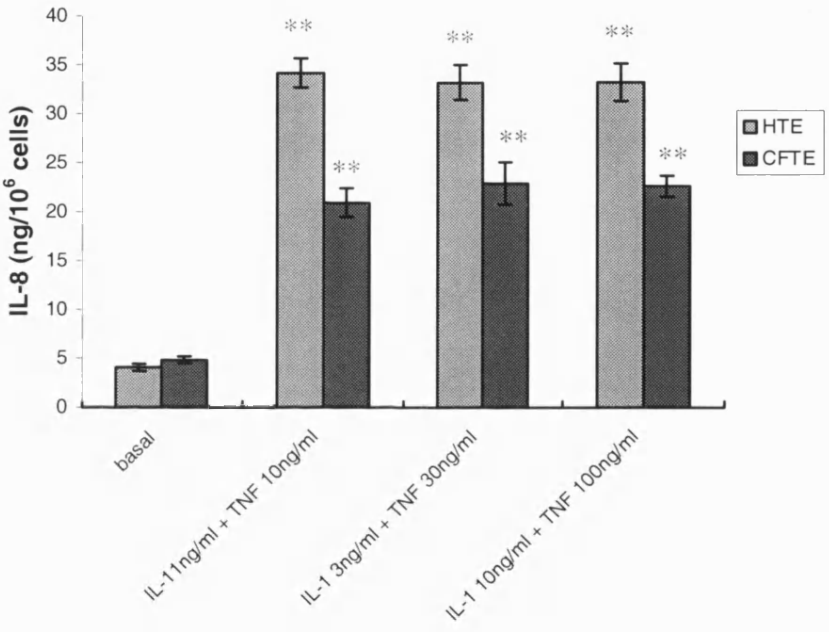


Figure 32 IL-8 production by 9HTEo$^-$ and ΣCFTE29o$^-$ cells after 24 h incubation at 37°C with IFNγ (100 units/ml), IL-1β (1-10 ng/ml) and TNFα (10-100 ng/ml) added in combination. The cells were serum starved for 12 hours prior to treatment. Basal is the amount of IL-8 produced in the presence of vehicle alone. Each bar is the mean ± SEM of three experiments (** $p<0.01$ compared with basal).

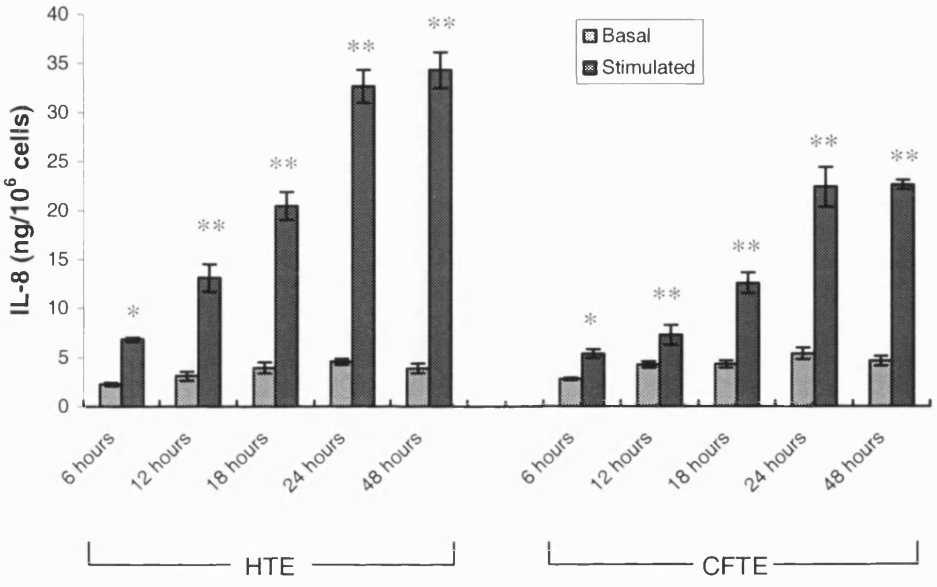


Figure 33 Time course of IL-8 production by 9HTEo$^-$ and ΣCFTE29o$^-$ cells after incubation at 37°C with vehicle or cytomix (IFNγ 100 units/ml, IL-1β 3 ng/ml and TNFα 30 ng/ml). The cells were serum starved for 12 hours prior to treatment. Each bar is the mean ± SEM of three experiments (* $p<0.05$, ** $p<0.01$ compared with basal).

conditions ($p<0.05$ for 6h and $p<0.01$ for 12-48h). To allow for valid comparisons between the IL-8 and the nitrite data obtained from these cell lines, all further stimulations were carried out over a 24-hour time period.

Figure 34 shows the effects of the protein synthesis inhibitor cycloheximide (10-100 μM) on IL-8 production by airway epithelial cells. This inhibitor had no effect on basal IL-8 levels, but it significantly reduced cytokine stimulated IL-8 generation in both cell lines. 30-minutes pre-treatment of 9HTEo$^-$ cells with 10 μM cycloheximide decreased cytokine stimulated IL-8 production from 35.97 ± 1.10 ng/10^6 cells to 8.60 ± 0.57 ng/10^6 cells ($p<0.01$). The use of 100 μM cycloheximide on this cell line further reduced cytokine stimulated IL-8 generation ($p<0.01$). This pattern was repeated for the ΣCFTE29o$^-$ cell line, with cytokine stimulated IL-8 production significantly reduced from 21.33 ± 1.81 ng/10^6 cells to 4.14 ± 0.29 ng/10^6 cells after pre-treatment with 100 μM cycloheximide ($p<0.01$).

To examine the possible modulatory effect of the phosphatidylinositol 3-kinase inhibitor LY294002 and IL-13 on IL-8 generation, airway epithelial cells were incubated with various concentrations of LY294002 (3-30 μM) and IL-13 (3-30 ng/ml) 30 minutes prior to stimulation with cytomix. LY294002 had no significant effect on IL-8 production by either cell line. This was also the case when unstimulated cells were treated with this inhibitor (Fig 35). Fig 36 shows that treatment of the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines with IL-13 also had no effect on IL-8 production in basal or stimulated conditions.

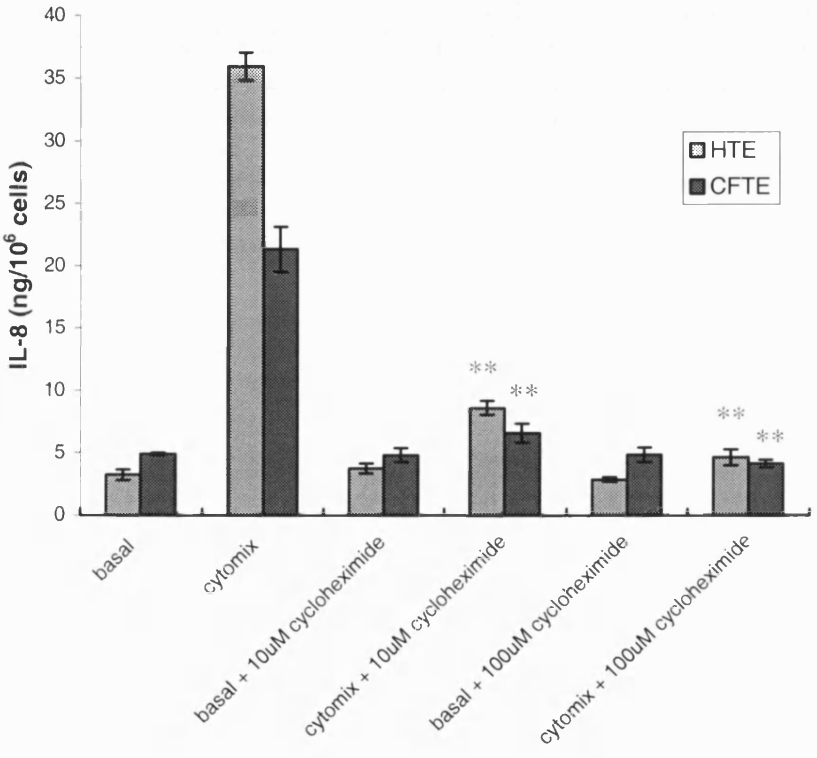


Figure 34 The effect of the protein synthesis inhibitor cycloheximide (10-100 μM) on IL-8 production by 9HTEo⁻ and ΣCFTE29o⁻ cells after 24 h incubation at 37°C with vehicle or cytomix (IFNγ 100 units/ml, IL-1β 3 ng/ml, TNFα 30 ng/ml). The cells were serum starved for 12 hours prior to treatment. Cycloheximide was added 30 minutes before the cytokines. Each bar is the mean ± SEM of three experiments (** $p<0.01$ compared with cytomix).

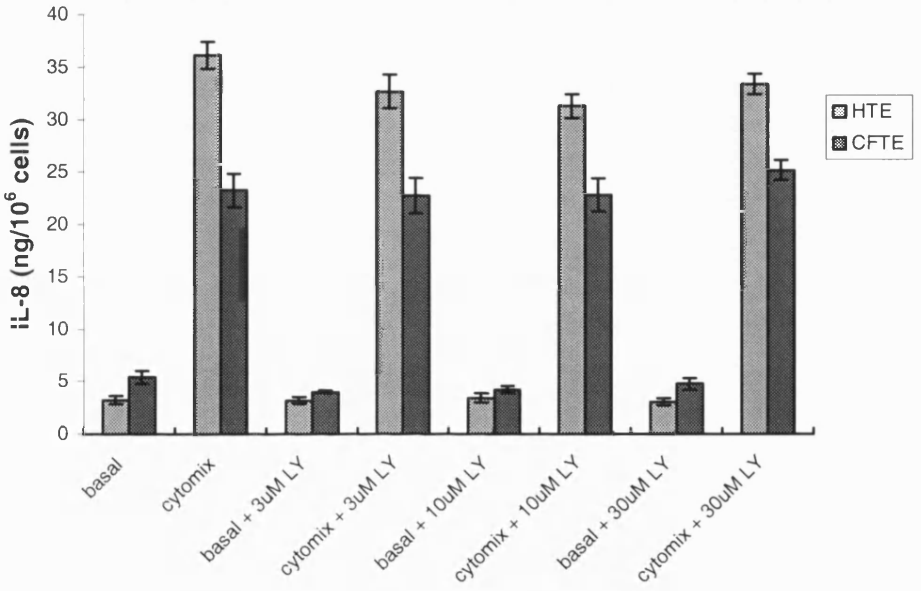


Figure 35 The effect of the phosphatidylinositol 3-kinase inhibitor LY294002 (3-30 μM) on IL-8 production by 9HTEo⁻ and ΣCFTE29o⁻ cells after 24 h incubation at 37°C with vehicle or cytomix (IFNγ 100 units/ml, IL-1β 3 ng/ml, TNFα 30 ng/ml). The cells were serum starved for 12 hours prior to treatment. LY294002 was added 30 minutes before the cytokines. Each bar is the mean ± SEM of three experiments.

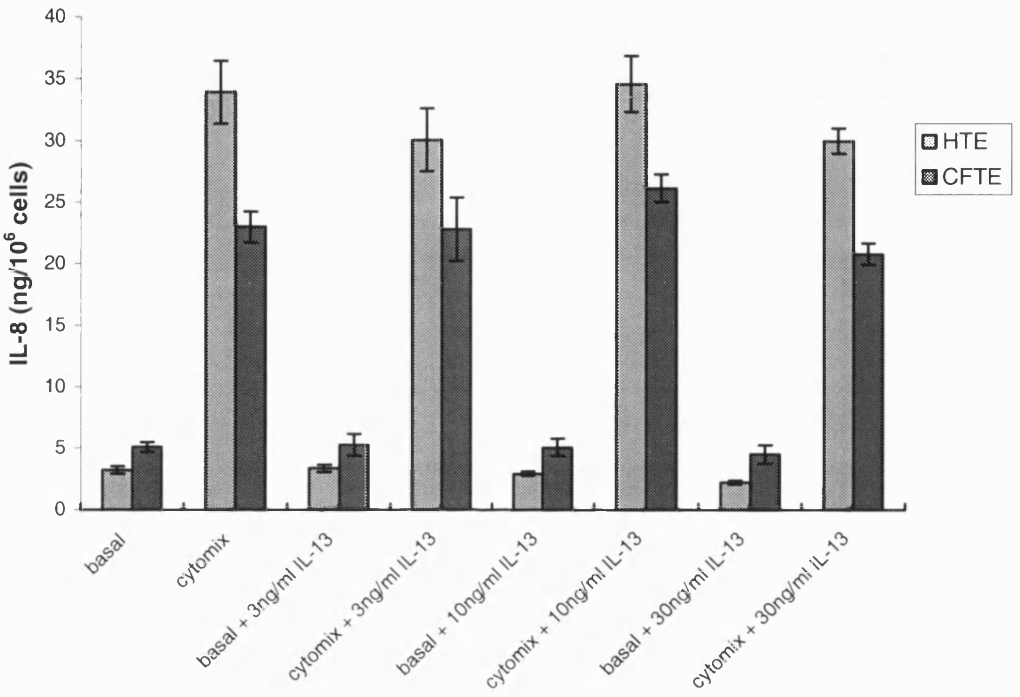


Figure 36 The effect of IL-13 (3-30 ng/ml) on IL-8 production by 9HTEo$^-$ and ΣCFTE29o$^-$ cells after 24 h incubation at 37°C with vehicle or cytomix (IFNγ 100 units/ml, IL-1β 3 ng/ml, TNFα 30 ng/ml). The cells were serum starved for 12 hours prior to treatment. IL-13 was added 30 minutes before the cytokines. Each bar is the mean ± SEM of three experiments.

4.3 INTERLEUKIN-8 GENERATION BY pCEP9HTEo⁻/#2 AND pCEP9HTEo⁻/RF CELLS

As with the 9HTEo⁻ and ΣCFTE29o⁻ cell lines both pCEP lines produced a small amount of IL-8 in the absence of cytokine stimulation (2.37 ± 0.25 ng/10^6 cells for pCEP9HTEo⁻/#2 cells and 3.47 ± 0.22 ng/10^6 cells for pCEP9HTEo⁻/RF cells) (Fig 37a), with the pCEP9HTEo⁻/RF cell line producing significantly more IL-8 than the pCEP9HTEo⁻/#2 cell line ($p<0.05$). Again, as with the 9HTEo⁻ and ΣCFTE29o⁻ cell lines, increasing concentrations of IFNγ had no effect on IL-8 generation compared with the basal levels of IL-8 produced by vehicle treated cells (Fig 37a), whereas increasing concentrations of IL-1β and TNFα added alone did affect IL-8 generation (Figs 37b & 38). IL-8 production by the 9HTEo⁻/pCEP#2 cell line was significantly increased upon incubation with IL-1β 1 ng/ml ($p<0.01$). Incubation of these cells with IL-1β 3 ng/ml further increased IL-8 generation, whereas incubation with IL-1β 10 ng/ml had no additional effects on IL-8 levels. Similarly, IL-8 production by the 9HTEo⁻/pCEPRF cell line significantly increased in the presence of IL-1β 1 ng/ml ($p<0.01$). Stimulation with IL-1β 3 ng/ml further increased IL-8 production and these levels were not significantly increased by stimulation of 9HTEo⁻/pCEPRF cells with IL-1β 10 ng/ml (Fig 37b). It is worth noting that at the higher concentrations of IL-1β (3 & 10 ng/ml) 9HTEo⁻/pCEPRF cells produced significantly more IL-8 than 9HTEo⁻/pCEP#2 cells ($p<0.05$). Stimulation of both cell lines with low concentrations of TNFα (3 & 10 ng/ml) produced a similar pattern of IL-8 production to that seen with the 9HTEo⁻ and ΣCFTE29o⁻ cells (Fig 38). Stimulations involving higher concentrations of TNFα (30 & 100 ng/ml) significantly increased IL-8 production by both cell lines ($p<0.01$), with levels rising to 10.11 ± 1.79 ng/10^6 cells for 9HTEo⁻/pCEP#2 cells stimulated with TNFα 100 ng/ml and to 11.68 ±

1.32 ng/10^6 cells for 9HTEo$^-$/pCEPRF cells stimulated with this concentration of TNFα. At these higher concentrations of TNFα no significant differences were found between the IL-8 levels produced by the two cell lines.

Stimulation of 9HTEo$^-$/pCEP#2 cells and 9HTEo$^-$/pCEPRF cells with combinations of IFNγ 100 units/ml and IL-1β (0-10 ng/ml) or TNFα (0-100 ng/ml) produced the same pattern of IL-8 generation as IL-1β or TNFα alone (Fig 39). Similarly, the combination of IL-1β (1-10 ng/ml) and TNFα (10-100 ng/ml) produced no significant differences in either cell line when compared to IL-1β alone (Fig 40). However, as with the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines, stimulation of the pCEP cells with increasing concentrations of the three proinflammatory cytokines combined did show an additive effect on IL-8 generation (Fig 41). Incubation of 9HTEo$^-$/pCEP#2 cells with IFNγ 100 units/ml, IL-1β 1 ng/ml and TNFα 10 ng/ml produced significantly more IL-8 (16.24 ± 1.22 ng/10^6 cells) than stimulation with IL-1β 1 ng/ml and TNFα 10 ng/ml (10.85 ± 1.16 ng/10^6 cells) ($p<0.05$). Stimulation of this cell line with higher concentrations of the three cytokines combined also resulted in significantly increased IL-8 production compared to corresponding concentrations of IL-1β and TNFα in combination ($p<0.05$). A similar pattern was observed with the 9HTEo$^-$/pCEPRF cell line; incubation with IFNγ 100 units/ml, IL-1β 1 ng/ml and TNFα 10 ng/ml lead to significantly increased IL-8 production (25.97 ± 1.27 ng/10^6 cells) compared to stimulation with IL-1β 1 ng/ml and TNFα 10 ng/ml (12.31 ± 1.18 ng/10^6 cells) ($p<0.01$). As with the 9HTEo$^-$/pCEP#2 cell line, stimulation of 9HTEo$^-$/pCEPRF cells with higher concentrations of the three cytokines combined also resulted in significantly increased IL-8 production compared to corresponding concentrations of IL-1β and TNFα in combination ($p<0.01$).

As previously, time course studies were carried out to investigate if stimulation of the airway epithelial cells with cytomix (IFNγ 100 units/ml, IL-1β 3 ng/ml and TNFα 30 ng/ml) resulted in induction of IL-8 at timepoints other than 24 hours. Once again a time-dependent increase in cytomix stimulated IL-8 production was observed for both cell lines. At each timepoint the amount of IL-8 produced by both cell lines upon cytokine stimulation was signifcantly greater than the amount produced under basal conditions ($p<0.05$ for 6h and $p<0.01$ for 12-48h). There was very little increase in basal IL-8 generation over time (Fig 42).

Table 2 summarises the production of cytokine stimulated IL-8 by 9HTEo$^-$/pCEP#2 and 9HTEo$^-$/pCEPRF cells after 30-minutes pre-treatment with cycloheximide, LY294002 or IL-13. Cycloheximide (10 μM) significantly decreased cytokine stimulated IL-8 production in 9HTEo$^-$/pCEP#2 cells from 16.70 ± 1.80ng/10^6 cells to 6.61 ± 0.43ng/10^6 cells ($p<0.01$). This pattern was repeated for the 9HTEo$^-$/pCEPRF cell line, with cytokine stimulated IL-8 production significantly reduced from 28.83 ± 2.02ng/10^6 cells to 10.56 ± 1.22ng/10^6 cells after pre-treatment with 10μM cycloheximide ($p<0.01$). Finally, neither the phosphatidylinositol 3-kinase inhibitor LY294002 nor IL-13 had any significant effect on cytokine stimulated IL-8 production by either cell line.

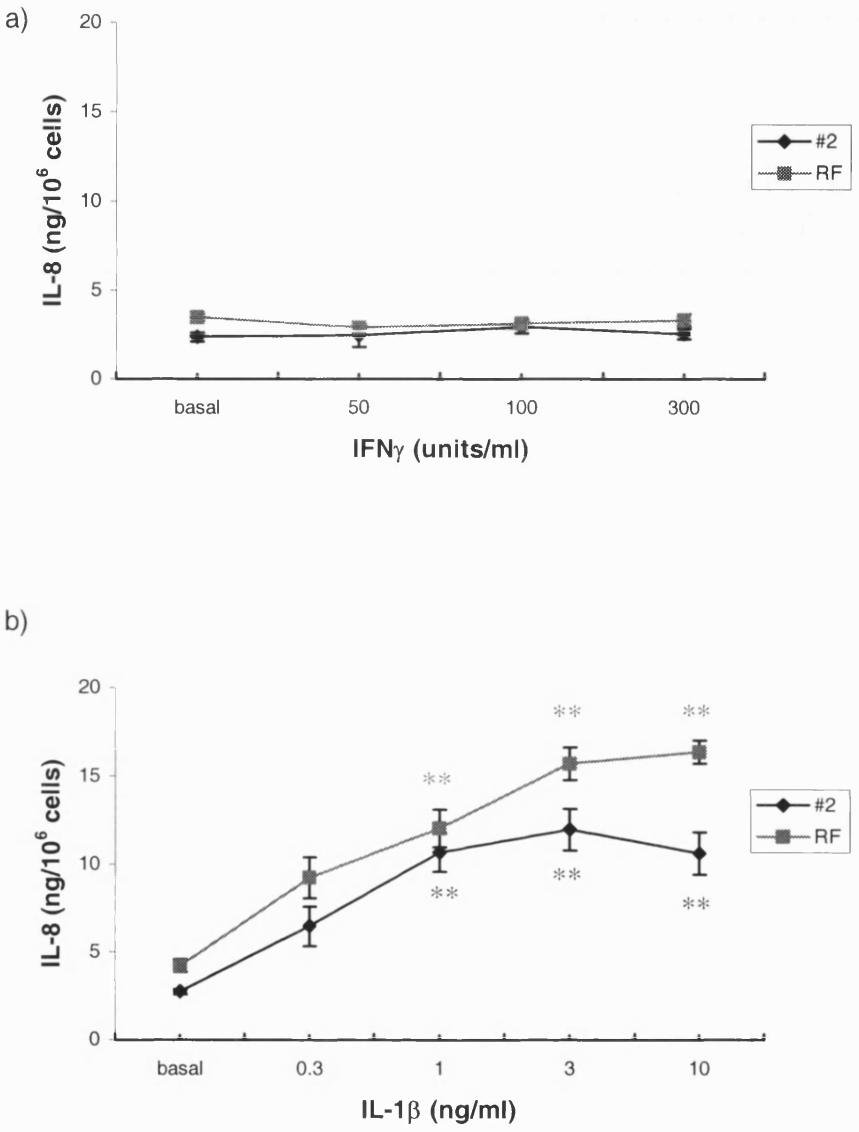


Figure 37 IL-8 production by 9HTEo$^-$/pCEP#2 and 9HTEo$^-$/pCEPRF cells after 24 h treatment at 37°C with (a) increasing concentrations of IFNγ (50-300 units/ml) and (b) increasing concentrations of IL-1β (0.3-10 ng/ml). The cells were serum starved for 12 hours prior to treatment. Basal is the amount of IL-8 produced in the presence of vehicle alone. Each point is the mean ± SEM of three experiments (** $p<0.01$ compared with basal).

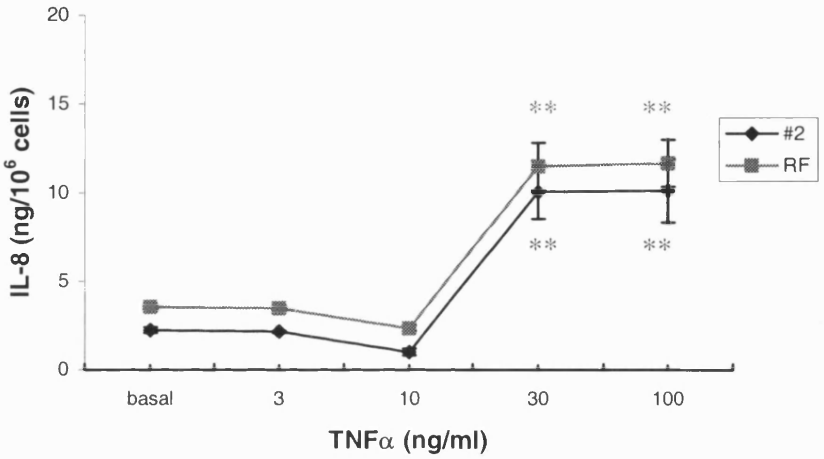


Figure 38 IL-8 production by 9HTEo⁻/pCEP#2 and 9HTEo⁻/pCEPRF cells after 24 h treatment at 37°C with increasing concentrations of TNFα (3-100 ng/ml). The cells were serum starved for 12 hours prior to treatment. Basal is the amount of IL-8 produced in the presence of vehicle alone. Each point is the mean ± SEM of three experiments (** $p<0.01$ compared with basal).

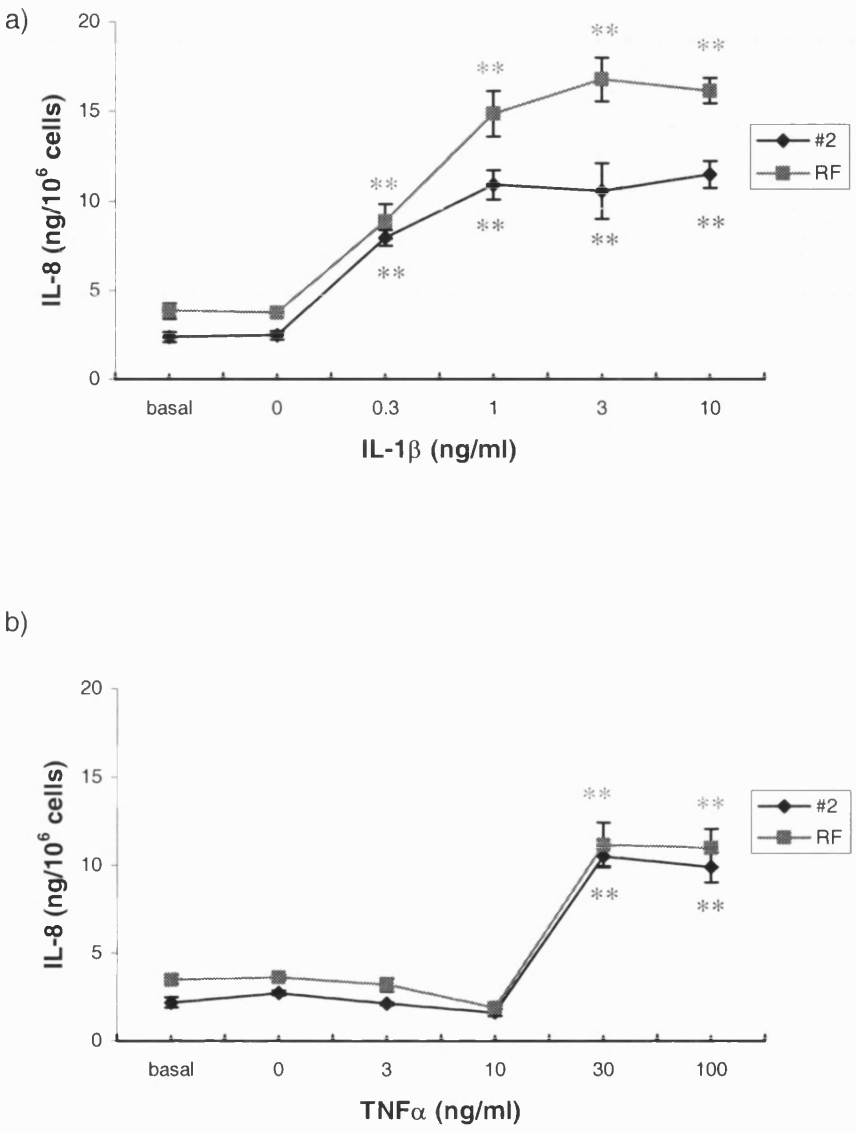


Figure 39 IL-8 production by 9HTEo⁻/pCEP#2 and 9HTEo⁻/pCEPRF cells after 24 h treatment at 37°C with (a) increasing concentrations of IL-1β (0-10 ng/ml) and (b) increasing concentrations of TNFα (0-100 ng/ml), in the presence of IFNγ 100 units/ml. The cells were serum starved for 12 hours prior to treatment. Basal is the amount of IL-8 produced in the presence of vehicle alone. Each point is the mean ± SEM of three experiments (** $p<0.01$ compared with basal).

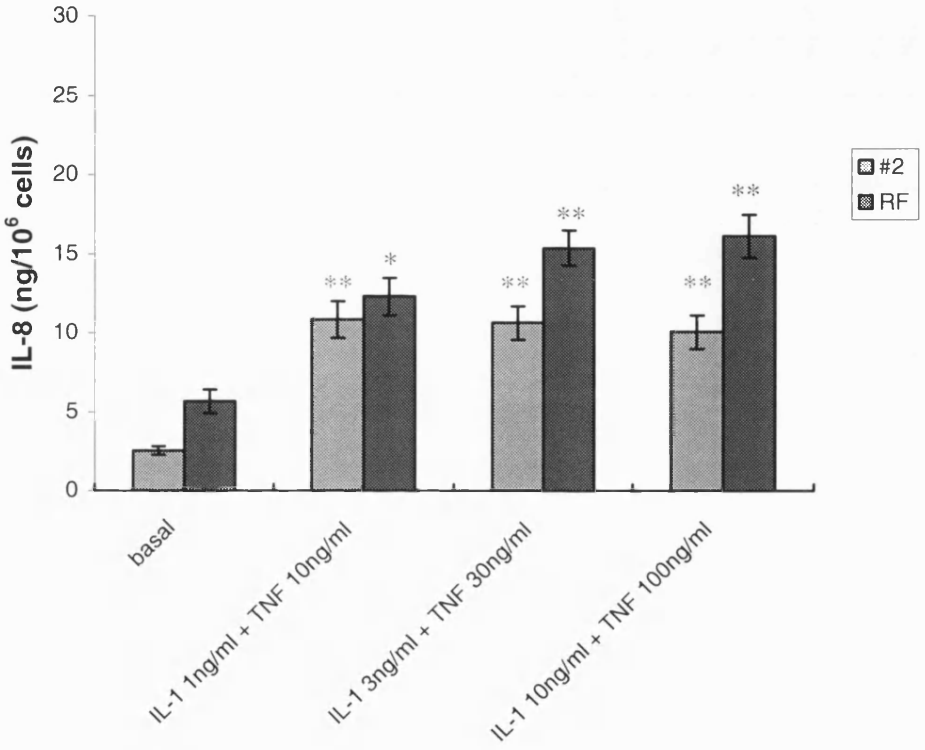


Figure 40 IL-8 production by 9HTEo⁻/pCEP#2 and 9HTEo⁻/pCEPRF cells after 24 h treatment at 37°C with IL-1β (1-10 ng/ml) and TNFα (10-100 ng/ml) added in combination. The cells were serum starved for 12 hours prior to treatment. Basal is the amount of IL-8 produced in the presence of vehicle alone. Each point is the mean ± SEM of three experiments (* $p<0.05$, ** $p<0.01$ compared with basal).

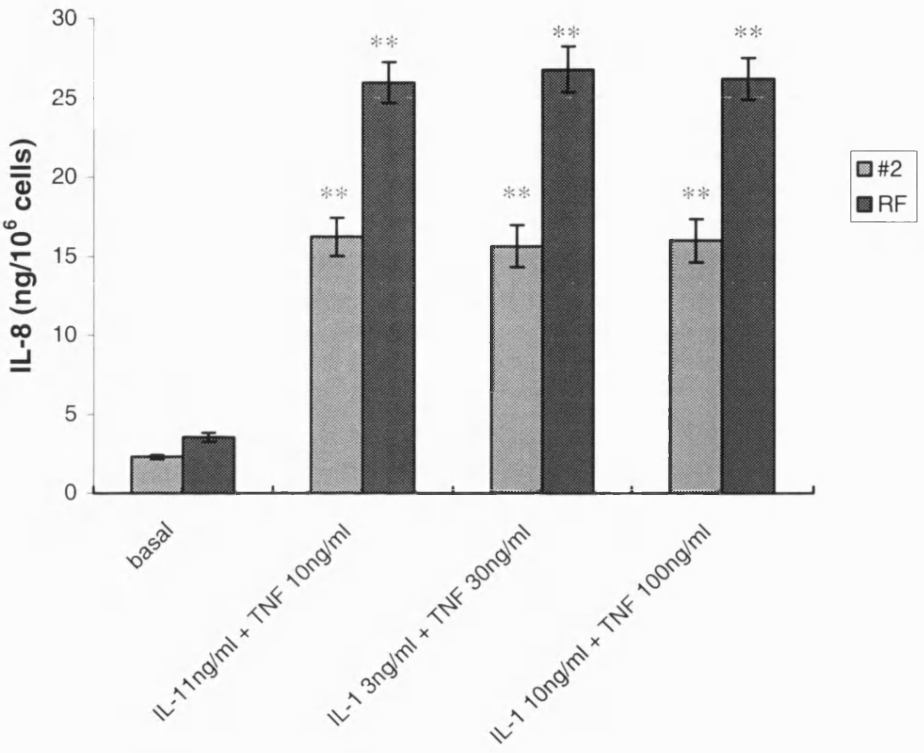


Figure 41 IL-8 production by 9HTEo$^-$/pCEP#2 and 9HTEo$^-$/pCEP#2 cells after 24 h incubation at 37°C with IFNγ (100 units/ml), IL-1β (1-10 ng/ml) and TNFα (10-100 ng/ml) added in combination. The cells were serum starved for 12 hours prior to treatment. Basal is the amount of IL-8 produced in the presence of vehicle alone. Each bar is the mean ± SEM of three experiments (** $p<0.01$ compared with basal).

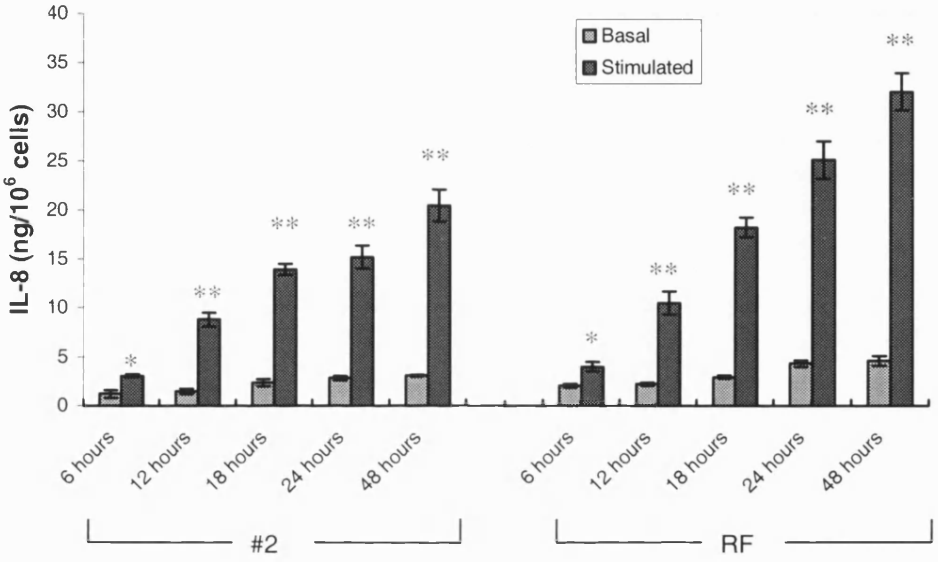


Figure 42 Time course of IL-8 production by 9HTEo⁻/pCEP#2 and 9HTEo⁻/pCEPRF cells after incubation at 37°C with vehicle or cytomix (IFNγ 100 units/ml, IL-1β 3 ng/ml and TNFα 30 ng/ml). The cells were serum starved for 12 hours prior to treatment. Each bar is the mean ± SEM of three experiments (* $p<0.05$, ** $p<0.01$ compared with basal).

9HTEo⁻/pCEP#2

Treatment	Mean ng IL-8/10^6 cells
basal	2.233 ± 0.17
cytomix	16.70 ± 1.80 **
cytomix + 10 μM cycloheximide	6.61 ± 0.43 **
cytomix + 10 μM LY294002	15.09 ± 0.95
cytomix + 30 ng/ml IL-13	17.05 ± 1.71

9HTEo⁻/pCEPRF

Treatment	Mean ng IL-8/10^6 cells
basal	3.95 ± 0.46
cytomix	28.83 ± 2.02 **
cytomix + 10 μM cycloheximide	10.56 ± 1.22 **
cytomix + 10 μM LY294002	27.26 ± 1.64
cytomix + 30 ng/ml IL-13	26.30 ± 1.94

Table 2 Summary of IL-8 production by 9HTEo⁻/pCEP#2 and 9HTEo⁻/pCEPRF cells after 24h treatment at 37°C with a variety of cytokine and inhibitor combinations. The cells were serum starved for 12 hours prior to treatment. Cytomix: IFNγ 100 units/ml, IL-1β 3 ng/ml, TNFα 30 ng/ml. Each value is the mean ± SEM of three experiments (** $p<0.01$ compared with control group).

4.4 DISCUSSION

Airway inflammation in CF is characterised by an excessive influx of neutrophils into the lung. Neutrophil chemotaxis is mediated primarily by the proinflammatory chemokine IL-8 (Baggiolini *et al.*, 1989) and elevated IL-8 levels have been detected in the bronchoalveolar lavage (BAL) fluid from CF patients (Richman-Eisenstat *et al.*, 1993). A potentially major source of IL-8 in the airways is the epithelium (Marini *et al.*, 1992; Becker *et al.*, 1993). Since the airway epithelium also manifests the most important phenotypic abnormalities of CF, many researchers have hypothesised that CF airway epithelial cells overproduce IL-8 and that this is linked to abnormal function of the cystic fibrosis transmembrane conductance regulator (CFTR). Several groups have demonstrated that stimulation of airway epithelial cell lines with *Pseudomonas aeruginosa*, leads to increased expression of IL-8 in cells with CFTR dysfunction compared to matched control lines (DiMango *et al.*, 1995; Bryan *et al.*, 1998; Tabary *et al.*, 1999). However, other studies examining the effects of cytokine stimulation on airway epithelial cells have produced conflicting data (Ruef *et al.*, 1993; Stecenko *et al.*, 1997; Black *et al.*, 1998; Schwiebert *et al.*, 1999; Massengale *et al.*, 1999). In order to successfully manage the excessive inflammation present in the CF lung, it will be important to accurately determine the source of increased IL-8 levels in CF airways and to discover the mechanisms controlling production of IL-8 by normal and CF airway cells. To attempt to clarify the role of airway epithelial cells in lung inflammation, part of this study was involved in examining the production and regulation of IL-8 in CF and non-CF airway epithelial cell lines.

The finding that all the cell lines used in this study produced a small amount of IL-8 in the absence of cytokine stimulation is consistent with other reports (Ruef *et al.*, 1993;

Stecenko *et al.*, 1997; Black *et al.*, 1998; Schwiebert *et al.*, 1999; Massengale *et al.*, 1999; Venkatakrishnan *et al.*, 2000). The ΣCFTE29o$^-$ and 9HTEo$^-$/pCEPRF cells produced more IL-8 than their non-CF counterparts, but this difference was only significant for the pCEP lines. As discussed previously, the pCEP cell lines are a genetically matched pair of cell lines and therefore any differences observed between them are potentially more valid. The fact that the 9HTEo$^-$/pCEPRF cell line produced more basal IL-8 than the 9HTEo$^-$/pCEP#2 cell line seems to suggest that CF airway epithelial cells are intrinsically more proinflammatory than non-CF airway epithelial cells and that this characteristic is linked to the absence of functional CFTR. However, although the differences in basal IL-8 levels between the pCEP lines are significant, they are still very small and the physiological relevance of such differences is questionable.

The significant increase in IL-8 levels observed when the cell lines were incubated with IL-1β or a high concentration of TNFα provides confirmation that airway epithelial cells are a possible source of IL-8 in the inflamed airway. The fact that IFNγ alone had no effect on basal IL-8 levels supports the findings of Schwiebert *et al.* (1999), who found either no effect or an inhibitory effect of IFNγ on IL-8 production with a variety of airway epithelial cell lines. Using gastric epithelial cell lines Yasumoto *et al.* (1992) demonstrated that although IFNγ had no effect on IL-8 production when used alone, co incubation of gastric cells with IFNγ and TNFα synergistically increased IL-8 production. Combining IFNγ and TNFα in this study resulted in no such synergism in any of the cell lines. However, a synergistic effect was observed when IFNγ, IL-1β and TNFα were used in combination as an inflammatory stimulus. IL-8 production has been investigated in many different experimental systems and its regulation occurs at a

variety of levels. Yasumoto and colleagues (1992) established that the cooperative effect of TNFα and IFNγ on IL-8 production in their gastric epithelial cell lines occurred at the level of transcription, through activation of the transcription factors NF-κB and AP-1. In 1994 Mukaida *et al.* stated that, depending on the cell line, cooperation between NF-κB and either NF-IL-6 or AP-1 is sufficient for IL-8 gene activation. In contrast, Smith *et al.* (2001) showed that when the airway epithelial cell line 16HBE14o$^-$ was stimulated with a combination of IL-1β and TNFα, IL-8 production occurred via the activation of NF-κB only. It is highly probable that the increased IL-8 levels produced by the 9HTEo$^-$, ΣCFTE29o$^-$, 9HTEo$^-$/pCEP#2 and 9HTEo$^-$/pCEPRF cell lines in response to IL-1β and TNFα stimulation involved activation of NF-κB. However, from the data gathered in this chapter, it is difficult to speculate on the exact mechanisms behind the synergistic effects of IFNγ, IL-1β and TNFα in these cell lines.

The response to cytokine stimulation over 48 hours showed similar patterns for the four cell lines studied. As reported in the previous chapter, at the 48 hour time point microscopic examination of the cells revealed a decrease in cell number and an increase in cell debris, with cell viability being 55% for the 9HTEo$^-$ cell line, 46% for the ΣCFTE29o$^-$ cell line, 62% for the 9HTEo$^-$/pCEP#2 cell line and 58% for the 9HTEo$^-$ /pCEPRF cell line. At all other time points cell viability was much higher and the amount of debris present was much less. In view of this, cytokine stimulations were routinely carried out over 24 hours. In contrast to the nitrite data, the cells did not appear to release large amounts of IL-8 after 48 hours. However, considering the state of the cell cultures at this time point, these IL-8 levels cannot be considered reliable. The fact that the protein synthesis inhibitor cycloheximide significantly reduced IL-8 production by all cell lines after a 24 hour cytokine stimulation, suggests that IL-8

generation is dependent on *de novo* protein synthesis and verifies the induction of IL-8 gene expression in response to proinflammatory cytokines. The inhibitor had no effect on basal IL-8 levels, confirming that there is always some constitutive IL-8 gene expression even in the absence of an inflammatory stimulus.

It has been demonstrated that the Th2 cytokine IL-13 modulates chemokine expression in a variety of cell types, including monocytes, endothelial cells and gut epithelial cells (Herbert *et al.*, 1993; Sironi *et al.*, 1994; Zurawski & De Vries, 1994; Kolios *et al.*, 1996). In the light of these findings and in view of the fact that IL-13 has been shown to inhibit NF-κB activation in some cell models (Manna & Aggarwai, 1998), it seemed reasonable to investigate a possible link between IL-13 and IL-8 production in airway epithelial cells. However, no effect of IL-13 on cytokine stimulated IL-8 expression was observed, suggesting there is no connection between IL-13, NF-κB and IL-8 gene expression in the 9HTEo$^-$ / ΣCFTE29o$^-$ and 9HTEo$^-$/pCEP#2 / 9HTEo$^-$/pCEPRF cell lines. The enzyme PI3K has also been implicated in NF-κB regulation in a range of cell lines (Ozes *et al.*, 1999; Romashkova & Makarov, 1999; Kane *et al.*, 1999; Madrid *et al.*, 2000) and the potential role of this enzyme in IL-8 production was investigated using the PI3K inhibitor LY294002. The finding that LY294002 had no effect on cytokine stimulated IL-8 levels appears to imply no link between PI3K and IL-8 production in these particular cell lines.

It is notable that the 9HTEo$^-$ cell line produced significantly more IL-8 than ΣCFTE29o$^-$ cell line in response to cytokine stimulation, whereas with the pCEP cell lines this pattern was reversed. There is evidence to support both these sets of findings. Venkatakrishnan *et al.* (2000) studied three different types of transformed human

bronchial epithelial cell lines. One type expressed mutant CFTR (IB3), one type expressed wild-type CFTR (BEAS-2B) and the third was a corrected CF cell line that was derived from IB3 cells stably transfected with wild-type CFTR (C38). They found that TNFα-stimulated production of IL-8 by IB3 cells was significantly increased compared with either BEAS-2B or C38 cells. These data attest to the hypothesis that cells with CFTR mutations over express IL-8 in response to proinflammatory signals and seem to support the results obtained with the pCEP cell lines. However, it is worth remembering that although the 9HTEo⁻/pCEPRF cells produced more IL-8 than the 9HTEo⁻/pCEP#2 cells after IL-1β and cytomix stimulation, when these lines were stimulated with TNFα alone, no significant differences in IL-8 production were observed. Ruef and co-workers (1993) demonstrated that the CF airway epithelial cell line JME/CF15 secreted IL-8 in the absence of stimuli and that this expression was further enhanced in the presence of IL-1β. In addition, Stecenko and colleagues (1997) reported that the CF airway epithelial cells IB3 and 2CF expressed significantly more IL-8 protein than the BEAS-2B cell line or normal primary bronchial epithelial cells in the presence of TNFα or IL-1β. These findings support the IL-β and cytomix results found with the pCEP cell lines. In contrast, other studies have shown no differences in the IL-8 response of CF and non-CF immortalised airway epithelial cell lines to cytokine stimulation (Black *et al.*, 1998; Schwiebert *et al.*, 1999; Massengale *et al.*, 1999). In fact, two of these studies actually demonstrated a trend towards lower IL-8 production in the cell lines with CFTR dysfunction (Black *et al.*, 1998; Massengale *et al.*, 1999). It is worth noting that both of these studies used isogenic cell lines and the authors claim this eliminates the possibility that the observed differences are due to inter-cell line variation. These findings refute the hypothesis that CF airway epithelial cells are primed to over express IL-8 and support the pattern of cytokine stimulated IL-8

production observed with the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines. Massengale and colleagues (1999) suggest their observations provide preliminary evidence that IL-8 secretion by CF respiratory epithelium is actually defective and propose this provides a potential mechanism for early airway colonisation with bacteria, particularly *P. aeruginosa*.

As the pCEP airway epithelial cell lines used in this particular study are a genetically matched pair of lines, the cytokine response profiles obtained from these cells may be more compelling than those obtained from the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines. However, in light of the studies discussed above it is hard to comment with any certainty on the relationship between non-functional CFTR and IL-8 production. It is difficult to explain the contradictory results obtained from all the studies on cytokine stimulated IL-8 production by airway epithelial cells. It should be noted that much of the work showing increased IL-8 production by CF airway epithelia did not report IL-8 secretion in a vector control line and those that used isogenic cells, did not demonstrate their corrected lines exhibited wild-type CFTR activity. On the other hand, many of the studies showing reduced IL-8 production by CF phenotype cells had inconsistencies in cell culture conditions. For example Massengale *et al.* (1999) cultured their CF cell lines in medium containing hydrocortisone and this glucocorticoid was not included in the normal cell line cultures. Hydrocortisone inhibits the transcription of inflammatory genes and this may well explain the differences seen.

Although the studies discussed here indicate that CF and non-CF airway epithelial cells express inconsistent differences in the levels of IL-8 produced in the presence and absence of cytokine stimulation, the mere expression of this chemokine implicates CF

epithelia at some level in the leukocyte migration observed in CF associated airway inflammation. Elevated levels of TNFα have been detected in the sputum of CF patients who were not infected with *Pseudomonas aeruginosa* (Schuster *et al*, 1995) and it may be that these increased TNFα levels contribute to the increased IL-8 levels detected in uninfected CF patient BAL fluid. However, at this stage it is impossible to claim with confidence that airway epithelial cells are the source of these increased IL-8 levels and that cytokine-induced epithelial IL-8 expression is dependent on functional CFTR.

4.5 SUMMARY OF RESULTS

- All cell lines produced a small amount of IL-8 in the absence of cytokine stimulation, with the 9HTEo$^-$/pCEPRF cell line producing significantly more IL-8 than the 9HTEo$^-$ /pCEP#2 cell line.
- IFNγ had no effect on basal IL-8 production, but IL-1β (3-10 ng/ml) or TNFα (30-100 ng/ml) alone significantly increased basal IL-8 levels in all cell lines.
- IL-1β and TNFα combined did not produce an additive effect on IL-8 production with any cell line.
- Although the addition of IFNγ to IL-1β or TNFα stimulations did not alter IL -8 generation, the combination of all 3 cytokines did have a synergistic effect on IL-8 levels produced by all cell lines.
- Upon cytokine stimulation (IL-1β alone, TNFα alone or cytomix), 9HTEo$^-$ cells produced significantly more IL-8 than ΣCFTE29o$^-$ cells.
- 9HTEo$^-$/pCEPRF cells produced significantly more IL-8 than 9HTEo$^-$/pCEP#2 cells after stimulation with IL-1β or cytomix.
- Differences in cytokine stimulated IL-8 levels between 9HTEo$^-$ and ΣCFTE29o$^-$ cells were significant at all time points (6-48 hours), but for the pCEP cell lines the differences were only significant at 18, 24 and 48 hours.
- Cycloheximide significantly reduced the cytokine stimulated IL-8 levels produced by all cell lines.
- Neither LY294002 nor IL-13 had any effect on the basal or cytokine stimulated IL-8 levels produced by any cell line.

5 PHOSPHATIDYLINOSITOL 3-KINASE SIGNALLING IN AIRWAY EPITHELIAL CELLS

5.1 INTRODUCTION

The aim of this work was to examine the mechanisms involved in the regulation of the phosphatidylinositol 3-kinase signal transduction pathway in CF and non-CF human airway epithelial cells, using the 9HTEo$^-$ (non-CF) and ΣCFTE29o$^-$ (CF) cell lines as models of human airway epithelium. PI3K activity was determined indirectly by measuring the levels of phosphorylated PKB and GSK3 (downstream effectors of PI3K) in stimulated and unstimulated cell lysates. The activity of PI3K was also examined directly by means of *in vitro* lipid kinase assays and finally the activity of the lipid phosphatase PTEN (an antagonist of PI3K signalling) was investigated using *in vitro* lipid phosphatase assays.

5.2 THE REGULATION OF PROTEIN KINASE B (PKB) AND GLYCOGEN SYNTHASE KINASE-3 (GSK3) PHOSPHORYLATION IN 9HTEo$^-$ AND ΣCFTE29o$^-$ CELLS

For experimental purposes, cells were seeded into petri dishes at 3-5 x 10^4 cells/ml in a total volume of 4 ml of complete medium and grown until they were approximately 90% confluent. The cells were then serum starved for 4 hours and subsequently stimulated at 37°C with vehicle or IL-13 (30 ng/ml) with or without 1 hour pretreatment with the PI3K inhibitor LY294002 (10 μM), the ERK1/2 inhibitor PD098059 (10 μM), the p38

inhibitor SB203580 (10 μM), the JNK inhibitor SP600125 (10 μM) and the protein kinase C (PKC) inhibitor Ro320432 (10 μM), either alone or in various combinations. Levels of phosphorylated PKB and GSK3 in cell lysates were determined via immunoblot analysis as described in the materials and methods section. Basal phosphorylated PKB levels were found to be low in both cell lines (Fig 43). Stimulation with the cytokine IL-13 (30 ng/ml) for 1-5 minutes had no detectable effect on phosphorylated PKB levels in either cell line. However by 15 minutes the levels of phosphorylated PKB had increased considerably to high levels in both cell lines (Fig 43). Pretreatment of the cells with the PI3K inhibitor LY294002 (10 μM) completely abrogated this response in both 9HTEo$^-$ and ΣCFTE29o$^-$ cells (Fig 43). Some phosphorylated GSK3 was detectable in the absence of cytokine stimulation in both cell lines (approximately 29% of maximum in 9HTEo$^-$ cells and 75% of maximum in ΣCFTE29o$^-$ cells) (Fig 44). As with phosphorylated PKB, these levels were increased to maximum levels after 15 minutes stimution with IL-13 (30 ng/ml). However, in this instance pretreatment of both cell lines with the PI3K inhibitor LY294002 (10 μM) only partially reduced the amount of phosphorylated GSK3 present (to approximately 60% and 42% of maximum after 20 minutes IL-13 stimulation for 9HTEo$^-$ cells and ΣCFTE29o$^-$ cells respectively) (Fig 44). It should be pointed out that the immunoblots show two bands for GSK3, which correspond to α and β isoforms of the protein. The β isoform has been studied much more intensively than the α isoform and therefore the lower band (GSK3β) was used when carrying out densitometry analysis and comparing phosphorylated GSK3 levels.

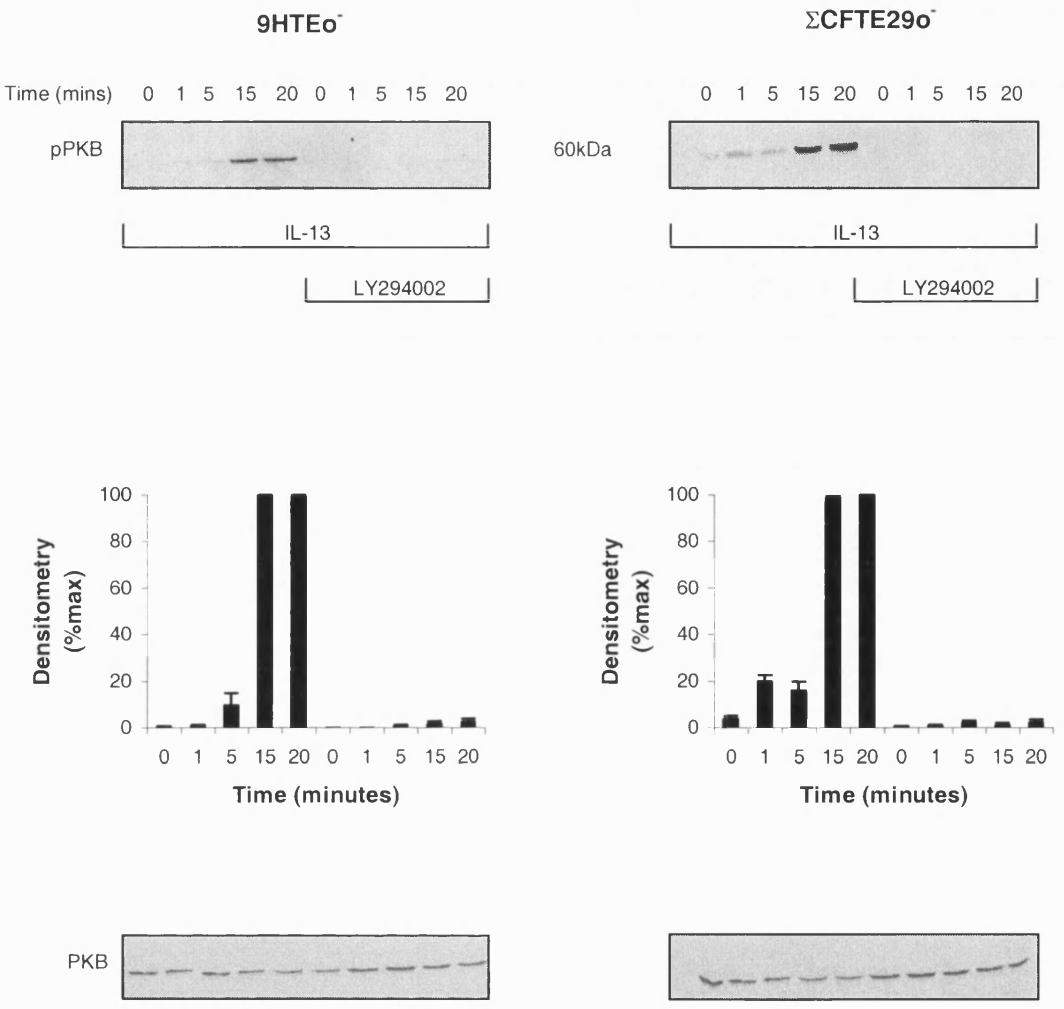


Figure 43 Phosphorylation of PKB in 9HTEo- and ΣCFTE29o- cells after stimulation with IL-13 (30 ng/ml) over a 20 minute time period at 37°C, +/- 1 hour pre-treatment with the PI3K inhibitor LY294002 (10 μM). The cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against phospho-(p) PKB (each blot is representative of 3 experiments), the middle panel is the densitometry analysis of the immunoblot (each bar is the mean ± SEM of 3 experiments) and the lower panel shows membranes probed with an antibody against pan PKB to check for equal loading.

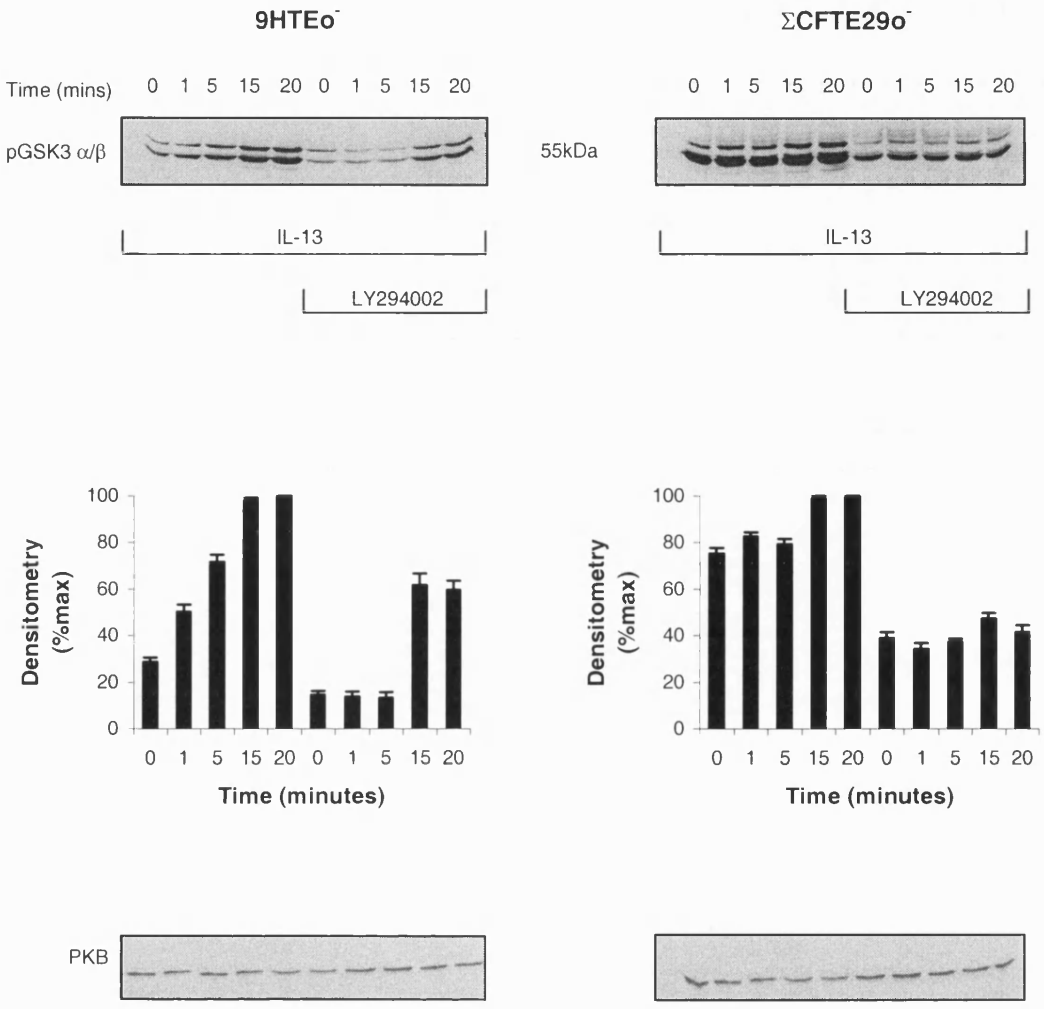


Figure 44 Phosphorylation of GSK3 in 9HTEo⁻ and ΣCFTE29o⁻ cells after stimulation with IL-13 (30 ng/ml) over a 20 minute time period at 37°C, +/- 1 hour pre-treatment with the PI3K inhibitor LY294002 (10 μM). The cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against phospho-(p) GSK3 (each blot is representative of 3 experiments), the middle panel is the densitometry analysis of the immunoblot (each bar is the mean ± SEM of 3 experiments) and the lower panel shows membranes probed with an antibody against pan PKB to check for equal loading.

Figure 45 shows the results of a time course study carried out to investigate the effects of IL-13 stimulation on phosphorylated PKB levels over a 90 minute period. This study supported the previous findings which showed low levels of phosphorylated PKB in both cell lines in the absence of cytokine stimulation (approximately 10% of maximum in 9HTEo$^-$ cells and 16% of maximum in ΣCFTE29o$^-$ cells) and demonstrated that PKB becomes phosphorylated within 10 minutes of IL-13 stimulation (to maximum levels in both cell lines). The levels of phosphorylated PKB remained consistently high throughout the 90 minute period and there were no observable differences between the two cell lines.

None of the inhibitors of the mitogen activated protein kinase (MAPK) pathway or the PKC inhibitor had any effect on IL-13 stimulated phosphorylated PKB levels in either cell line (Figs 46-49). However, some of these inhibitors did have effects on phosphorylated GSK3 levels (Figs 50-53). For example, the ERK1/2 inhibitor PD098959 (10 μM) did not appear to reduce basally phosphorylated GSK3 levels in 9HTEo$^-$ cells but did reduce basal phosphorylated GSK3 levels in ΣCFTE29o$^-$ cells from 50% of maximum to 33% (Fig 50). When considering the 20-minute time point, the levels of phosphorylated GSK3 in 9HTEo$^-$ cells were reduced to 82% of maximum and in ΣCFTE29o$^-$ cells to 21% of maximum (Fig. 50). In contrast, the use of the p38 inhibitor SB203580 (10 μM) had no effect on phosphorylated GSK3 levels in either cell line (Fig 51). The JNK inhibitor SP600125 (10 μM) appeared to have little effect on the levels of phosphorylated GSK3 in 9HTEo$^-$ cells, but did have an effect on phosphorylated GSK3 levels in the ΣCFTE29o$^-$ cell line (Fig 52). In the absence of cytokine stimulation SP600125 reduced phosphorylated GSK3 levels in these cells from approximately 30% of maximum to 15% and at the 15 minute time point it decreased

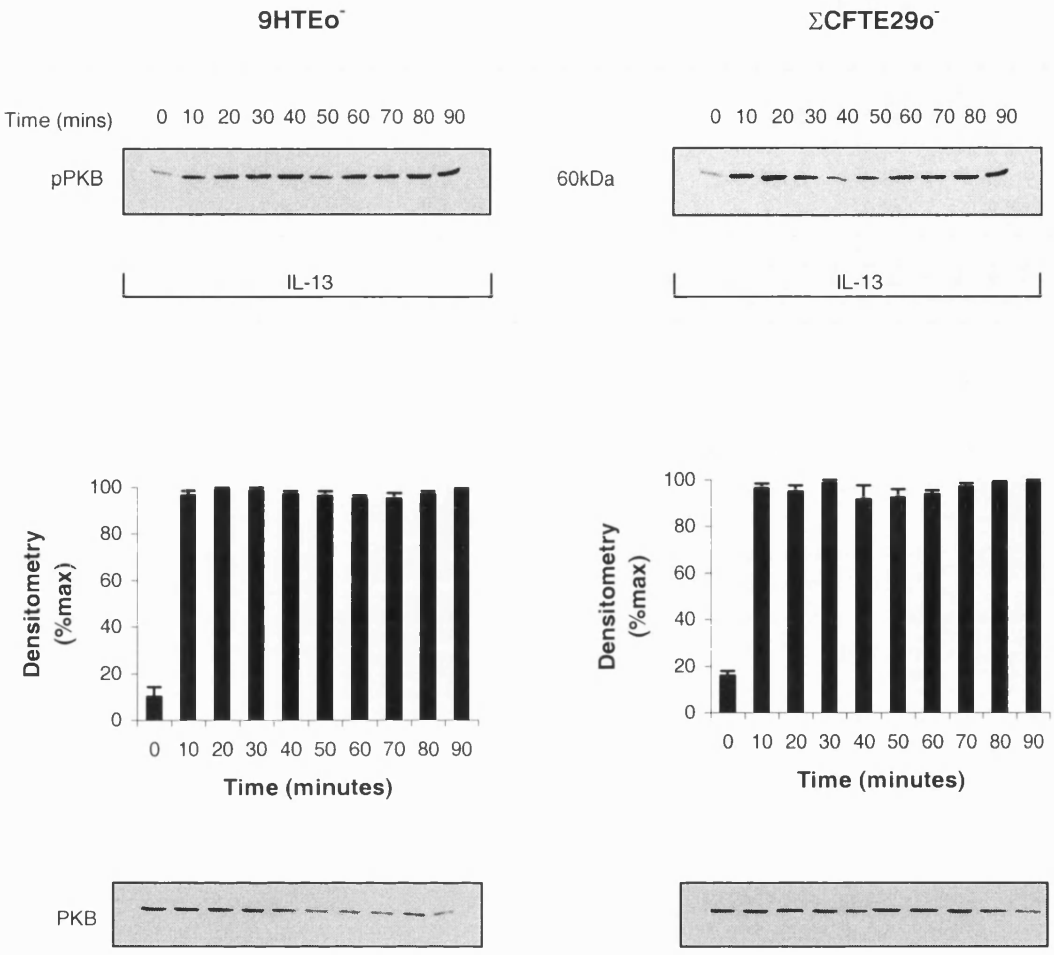


Figure 45 Phosphorylation of PKB in 9HTEo⁻ and ΣCFTE29o⁻ cells after stimulation with IL-13 (30 ng/ml) over a 90 minute time period at 37°C. The cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against phospho-(p) PKB (each blot is representative of 3 experiments), the middle panel is the densitometry analysis of the immunoblot (each bar is the mean ± SEM of 3 experiments) and the lower panel shows membranes probed with an antibody against pan PKB to check for equal loading.

the levels of phosphorylated GSK3 from approximately 96% of maximum to 32%. Finally the use of the PKC inhibitor Ro320432 (10 μM) also had differing effects on phosphorylated GSK3 levels in the two cell lines (Fig 53). In the 9HTEo$^-$ cell line Ro320432 decreased basally phosphorylated GSK3 levels from approximately 54% of maximum to 20% and at the 15-minute time point it had the effect of reducing the amount of IL-13 stimulated phosphorylated GSK3 from maximum levels to 24% of maximum. By contrast, in the ΣCFTE29o$^-$ cell line the PKC inhibitor reduced basally phosphorylated GSK3 levels from approximately 67% of maximum to 2% and reduced IL-13 stimulated levels from approximately 100% to 6% (15-minute time point). It is also interesting to note that the ERK1/2 inhibitor (PD098059), the JNK inhibitor (SP600125) and the PKC inhibitor (Ro320432) all substantially decreased the levels of phosphorylated GSK3α in the ΣCFTE29o$^-$ cell line whilst having little effect on GSK3α levels in the 9HTEo$^-$ cell line (Figs 50, 52 & 53).

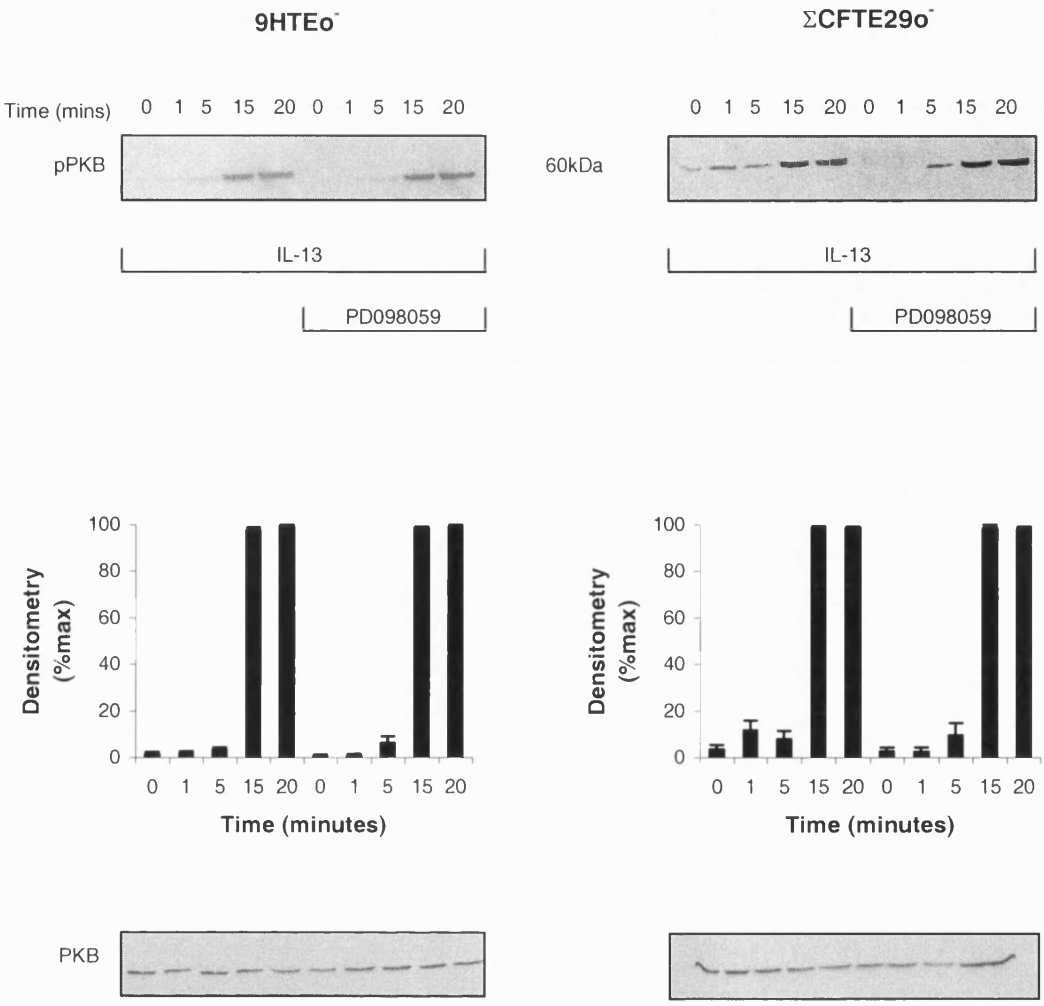


Figure 46 Phosphorylation of PKB in 9HTEo⁻ and ΣCFTE29o⁻ cells after stimulation with IL-13 (30 ng/ml) over a 20 minute time period at 37°C, +/- 1 hour pre-treatment with the ERK1/2 inhibitor PD098059 (10 μM). The cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against phospho-(p) PKB (each blot is representative of 3 experiments), the middle panel is the densitometry analysis of the immunoblot (each bar is the mean ± SEM of 3 experiments) and the lower panel shows membranes probed with an antibody against pan PKB to check for equal loading.

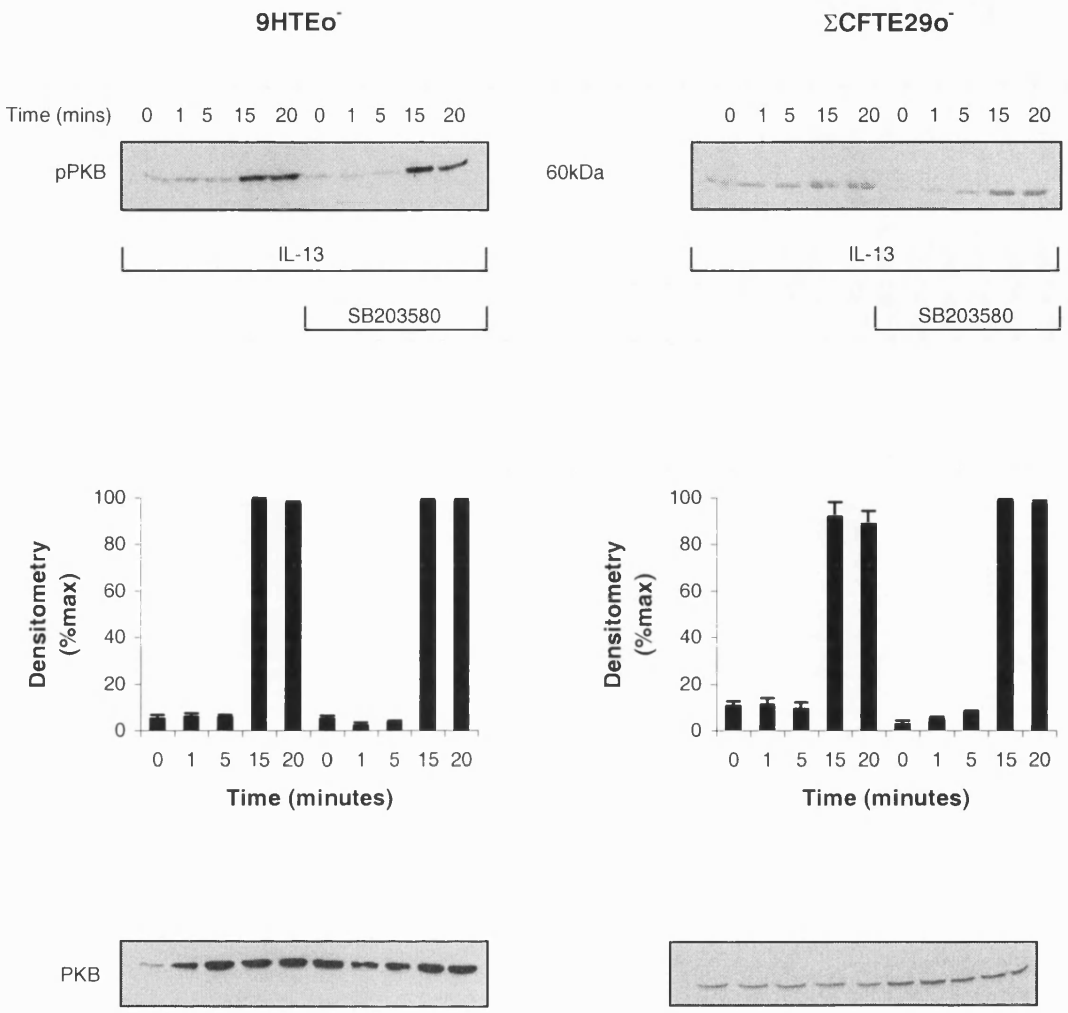


Figure 47 Phosphorylation of PKB in 9HTEo⁻ and ΣCFTE29o⁻ cells after stimulation with IL13 (30 ng/ml) over a 20 minute time period at 37°C, +/- 1 hour pre-treatment with the p38 inhibitor SB203580 (10 μM). The cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against phospho-(p) PKB (each blot is representative of 3 experiments), the middle panel is the densitometry analysis of the immunoblot (each bar is the mean ± SEM of 3 experiments) and the lower panel shows membranes probed with an antibody against pan PKB to check for equal loading.

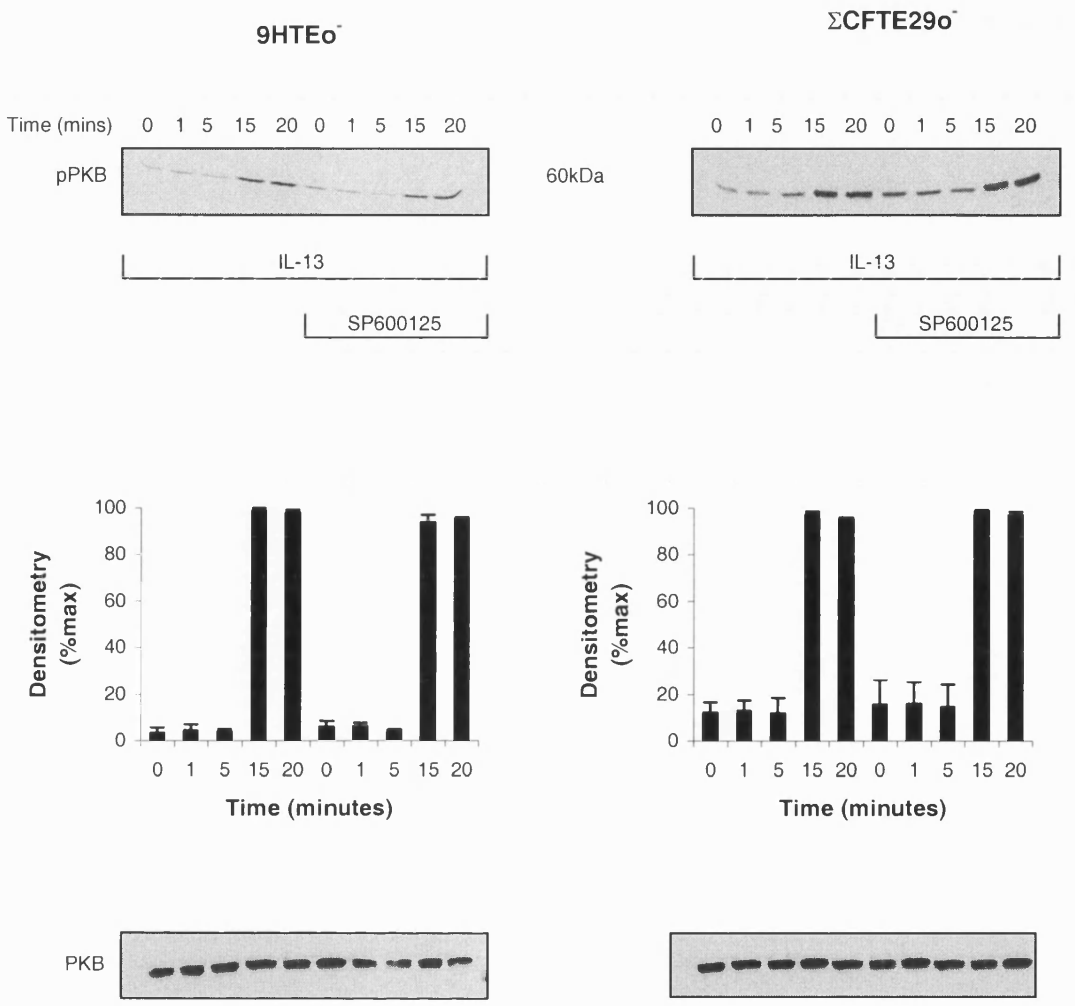


Figure 48 Phosphorylation of PKB in 9HTEo- and ΣCFTE29o- cells after stimulation with IL-13 (30 ng/ml) over a 20 minute time period at 37°C, +/- 1 hour pre-treatment with the JNK inhibitor SP600125 (10 μM). The cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against phospho-(p) PKB (each blot is representative of 3 experiments), the middle panel is the densitometry analysis of the immunoblot (each bar is the mean ± SEM of 3 experiments) and the lower panel shows membranes probed with an antibody against pan PKB to check for equal loading.

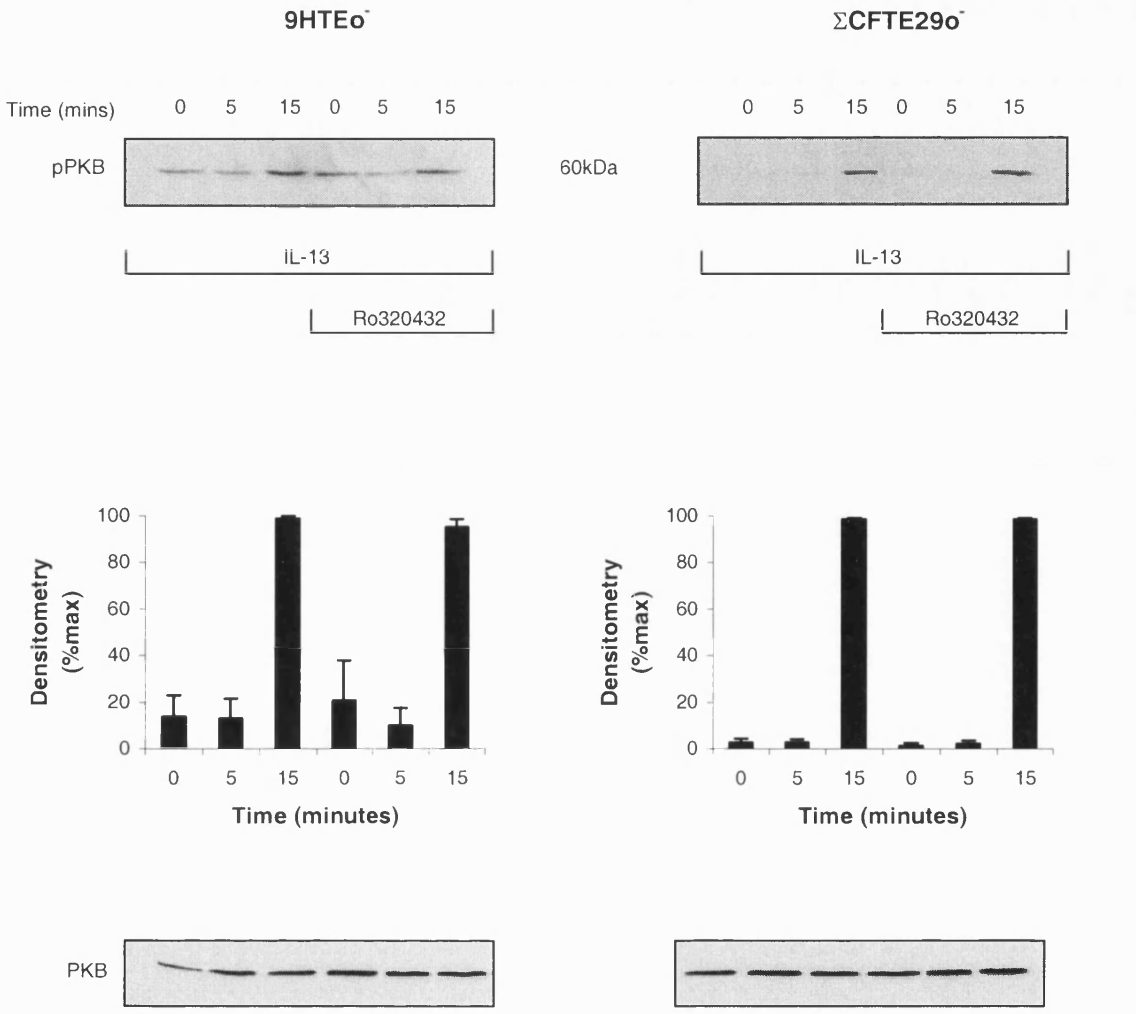


Figure 49 Phosphorylation of PKB in 9HTEo- and ΣCFTE29o- cells after stimulation with IL-13 (30 ng/ml) over a 15 minute time period at 37°C, +/- 1 hour pre-treatment with the PKC inhibitor Ro320432 (10 μM). The cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against phospho-(p) PKB (each blot is representative of 3 experiments), the middle panel is the densitometry analysis of the immunoblot (each bar is the mean ± SEM of 3 experiments) and the lower panel shows membranes probed with an antibody against pan PKB to check for equal loading.

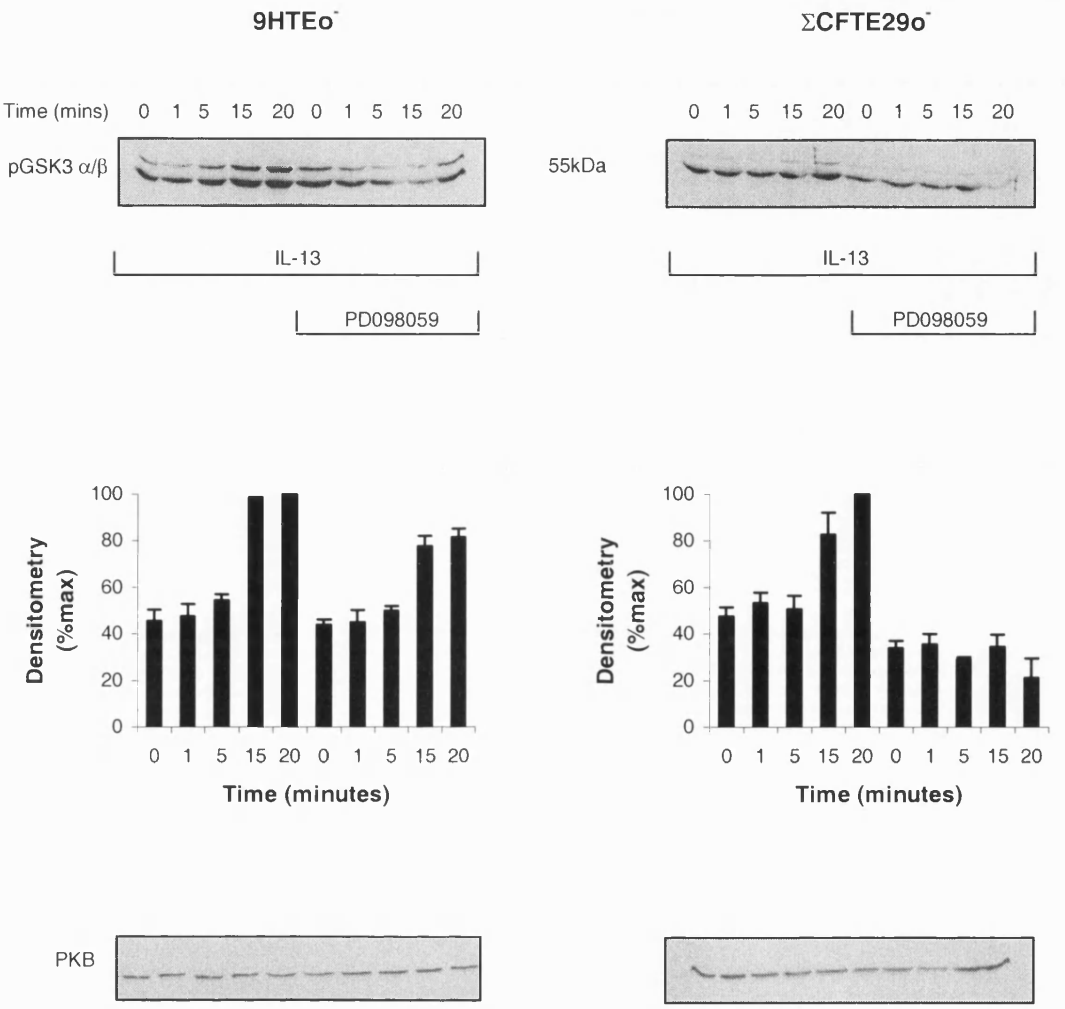


Figure 50 Phosphorylation of GSK3 in 9HTEo⁻ and ΣCFTE29o⁻ cells after stimulation with IL-13 (30 ng/ml) over a 20 minute time period at 37°C, +/- 1 hour pre-treatment with the ERK1/2 inhibitor PD098059 (10 μM). The cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against phospho-(p) GSK3 (each blot is representative of 3 experiments), the middle panel is the densitometry analysis of the immunoblot (each bar is the mean ± SEM of 3 experiments) and the lower panel shows membranes probed with an antibody against pan PKB to check for equal loading.

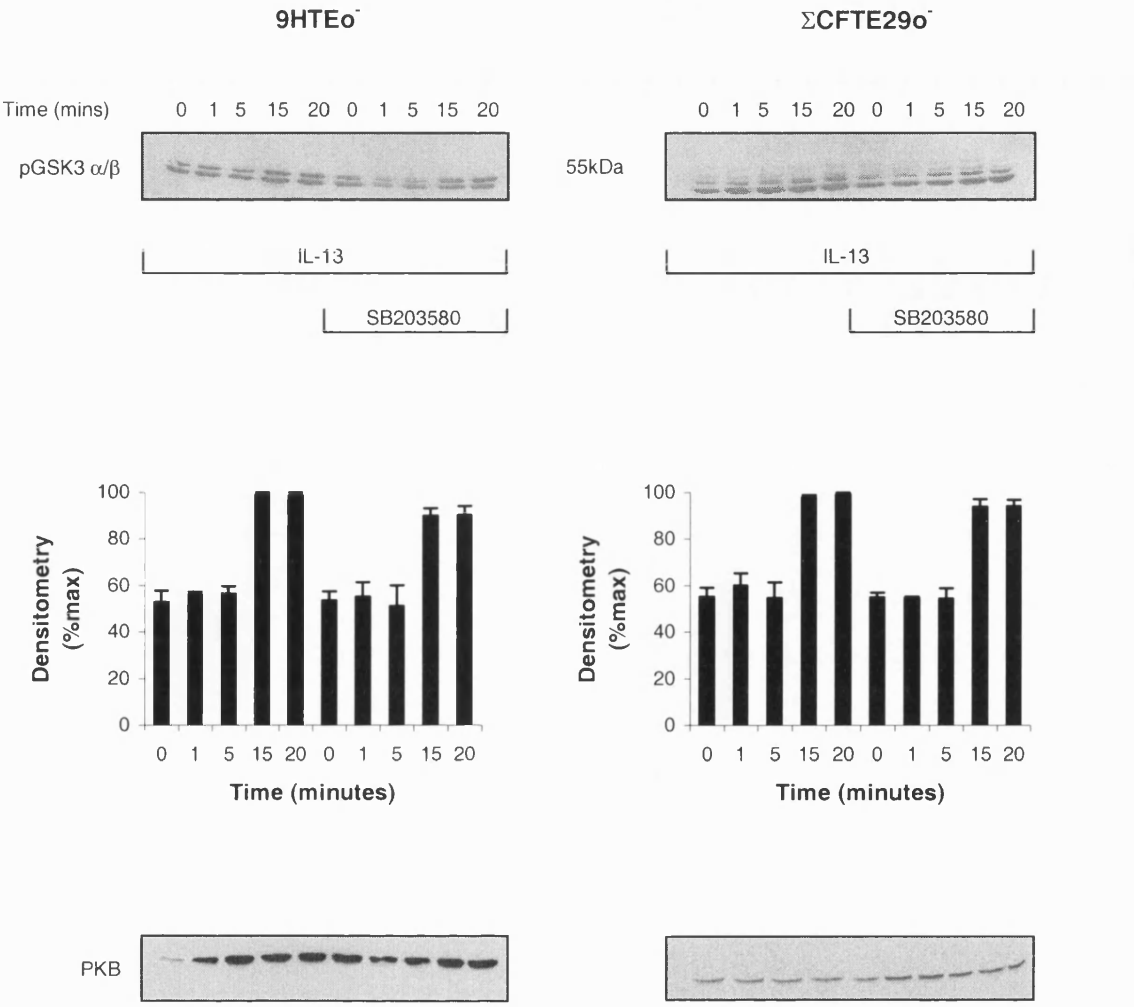


Figure 51 Phosphorylation of GSK3 in 9HTEo- and ΣCFTE29o- cells after stimulation with IL-13 (30 ng/ml) over a 20 minute time period at 37°C, +/- 1 hour pre-treatment with the p38 inhibitor SB203580 (10 μM). The cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against phospho-(p) GSK3 (each blot is representative of 3 experiments), the middle panel is the densitometry analysis of the immunoblot (each bar is the mean ± SEM of 3 experiments) and the lower panel shows membranes probed with an antibody against pan PKB to check for equal loading.

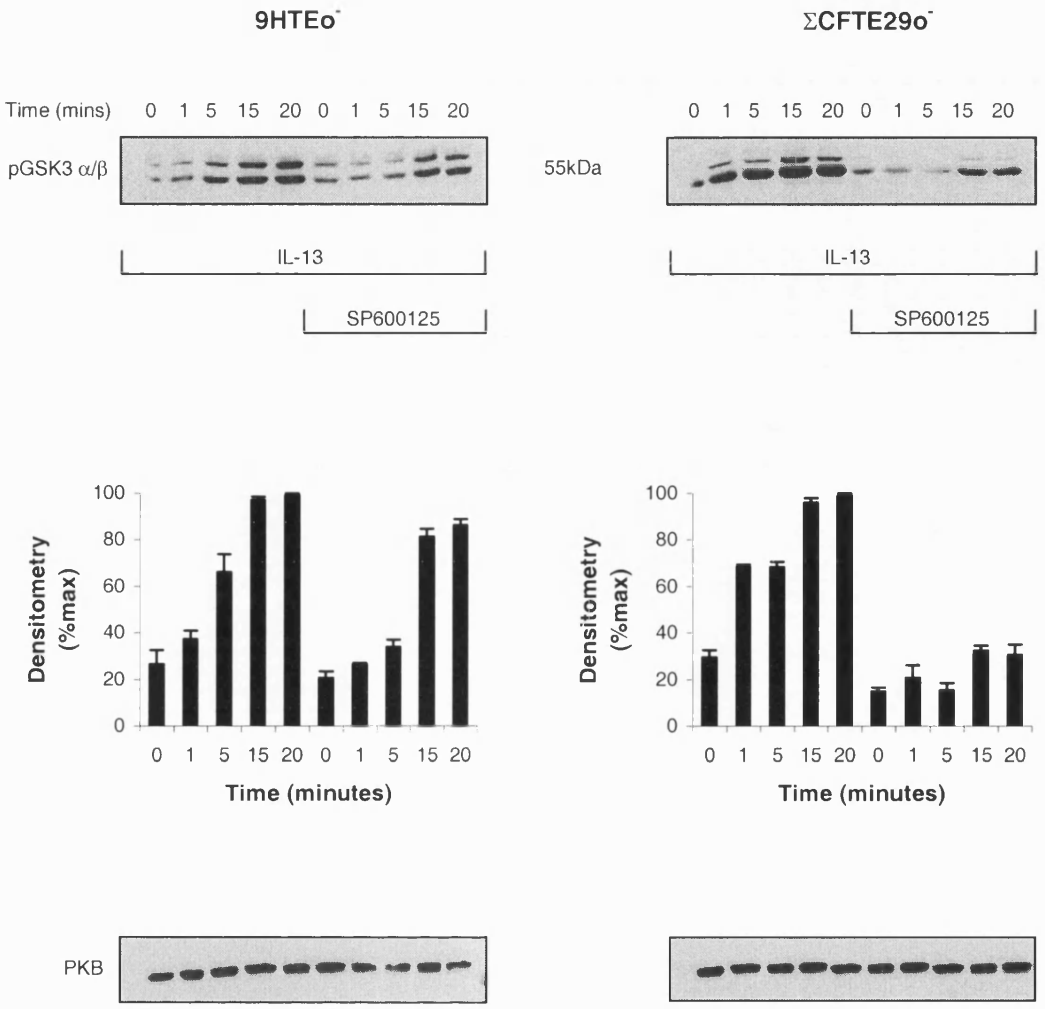


Figure 52 Phosphorylation of GSK3 in 9HTEo- and ΣCFTE29o- cells after stimulation with IL-13 (30 ng/ml) over a 20 minute time period at 37°C, +/- 1 hour pre-treatment with the JNK inhibitor SP600125 (10 μM). The cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against phospho-(p) GSK3 (each blot is representative of 3 experiments), the middle panel is the densitometry analysis of the immunoblot (each bar is the mean ± SEM of 3 experiments) and the lower panel shows membranes probed with an antibody against pan PKB to check for equal loading.

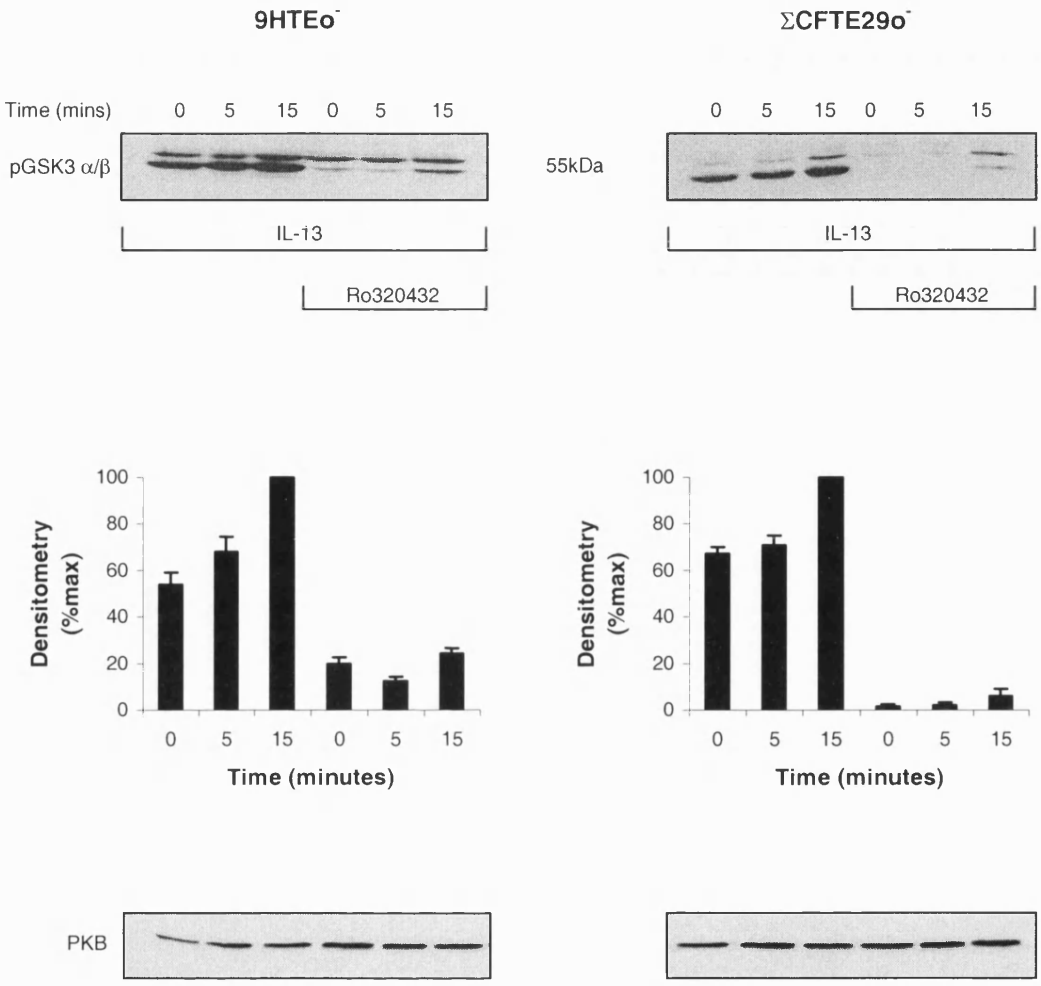


Figure 53 Phosphorylation of GSK3 in 9HTEo⁻ and ΣCFTE29o⁻ cells after stimulation with IL-13 (30 ng/ml) over a 15 minute time period at 37°C, +/- 1 hour pre-treatment with the PKC inhibitor Ro320432 (10 μM). The cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against phospho-(p) GSK3 (each blot is representative of 3 experiments), the middle panel is the densitometry analysis of the immunoblot (each bar is the mean ± SEM of 3 experiments) and the lower panel shows membranes probed with an antibody against pan PKB to check for equal loading.

The pattern of phosphorylated GSK3 inhibition observed when using the MAPK and PKC inhibitors alone was repeated when these inhibitors were used in combination (Figs 54-56). When the PI3K inhibitor LY294002 (10 μM) was used in conjunction with the ERK1/2 inhibitor PD098059 (10 μM), the resulting pattern of GSK3 phosphorylation in the 9HTEo$^-$ cell line (Fig 54) was very similar to that obtained with the use of LY294002 alone. In contrast when these two inhibitors were used with the ΣCFTE29o$^-$ cell line (Fig 54), PD098059 appeared to have an additive effect on phosphorylated GSK3 inhibition compared with LY294002 alone. In fact the combination of these two inhibitors reduced basally phosphorylated GSK3 levels in ΣCFTE29o$^-$ cells from approximately 16% of maximum to 7% and reduced IL-13 stimulated levels from approximately 100% to 1% (15-minute time point). The combination of LY294002 (10 μM) and the JNK inhibitor SP600125 (10 μM) also had a greater inhibitory effect on phosphorylated GSK3 in the ΣCFTE29o$^-$ cell line compared with the 9HTEo$^-$ cell line (Fig 55). Levels of phosphorylated GSK3 after 15 minutes IL-13 stimulation were reduced from maximal levels to approximately 52% of maximum in 9HTEo$^-$ cells and from approximately 99% to 6 % in ΣCFTE29o$^-$ cells. When LY294002 (10 μM) and the PKC inhibitor Ro320432 (10 μM) were used in combination, phosphorylation of GSK3 was almost completely inhibited in both cell lines (Fig 56). In the 9HTEo$^-$ cell line levels of basally phosphorylated GSK3 were reduced from approximately 63% of maximum to 5% and in the ΣCFTE29o$^-$ cell line these levels were reduced from approximately 44% of maximum to 1%. At the 15-minute time point, phosphorylated GSK3 levels were reduced from near maximum to 4% of maximum in 9HTEo$^-$ cells and from 100% to 0% in ΣCFTE29o$^-$ cells.

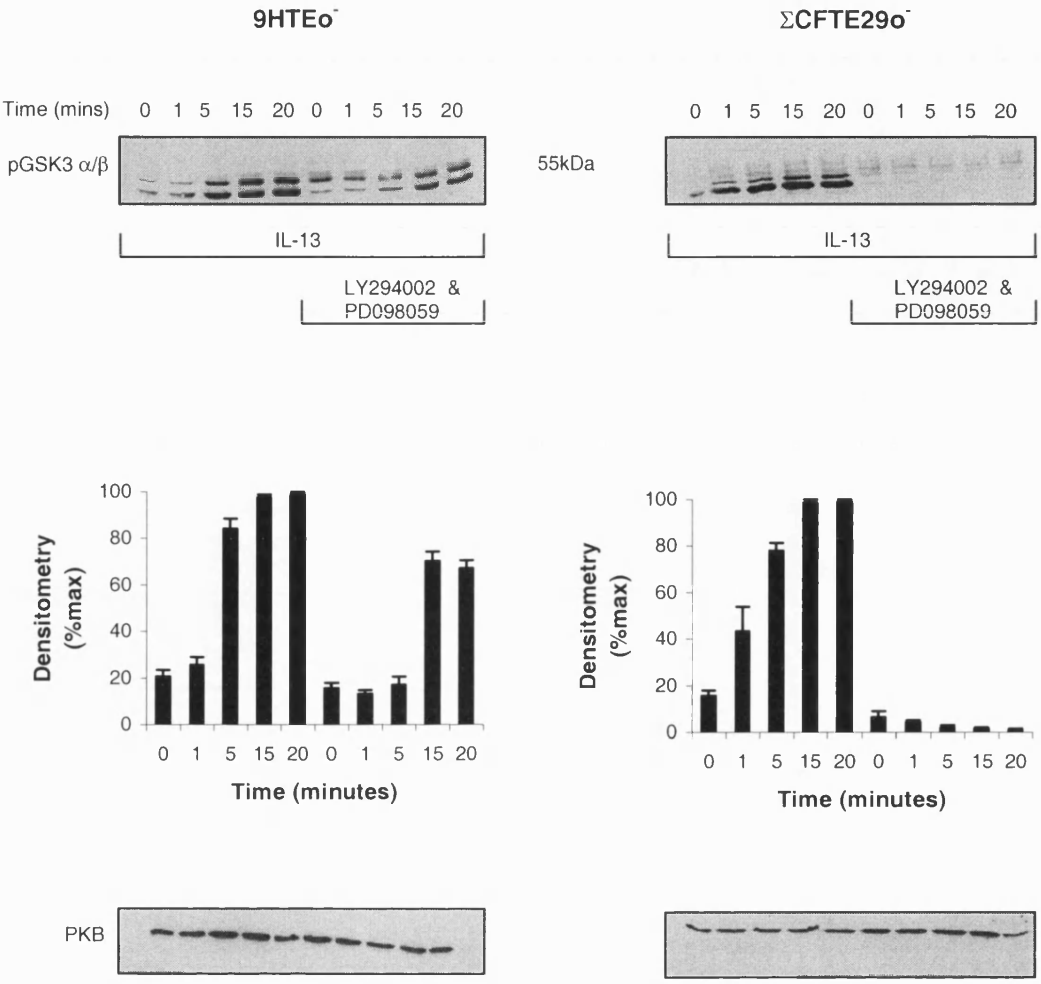


Figure 54 Phosphorylation of GSK3 in 9HTEo- and ΣCFTE29o- cells after stimulation with IL-13 (30 ng/ml) over a 20 minute time period at 37°C, +/- 1 hour pre-treatment with the PI3K inhibitor LY294002 (10 μM) and the ERK1/2 inhibitor PD098059 (10 μM). The cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against phospho-(p) GSK3 (each blot is representative of 3 experiments), the middle panel is the densitometry analysis of the immunoblot (each bar is the mean ± SEM of 3 experiments) and the lower panel shows membranes probed with an antibody against pan PKB to check for equal loading.

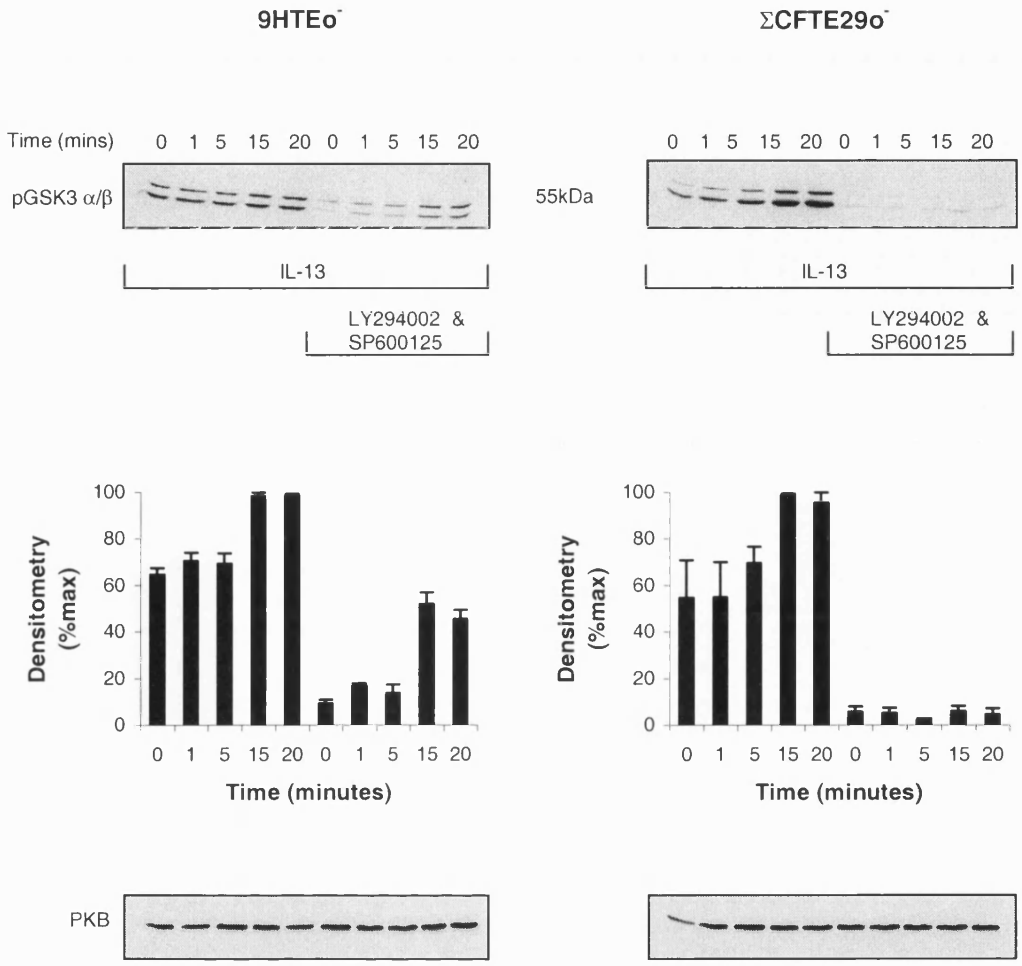


Figure 55 Phosphorylation of GSK3 in 9HTEo- and ΣCFTE29o- cells after stimulation with IL-13 (30 ng/ml) over a 20 minute time period at 37°C, +/- 1 hour pre-treatment with the PI3K inhibitor LY294002 (10 μM) and the JNK inhibitor SP600125 (10 μM). The cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against phospho-(p) GSK3 (each blot is representative of 3 experiments), the middle panel is the densitometry analysis of the immunoblot (each bar is the mean ± SEM of 3 experiments) and the lower panel shows membranes probed with an antibody against pan PKB to check for equal loading.

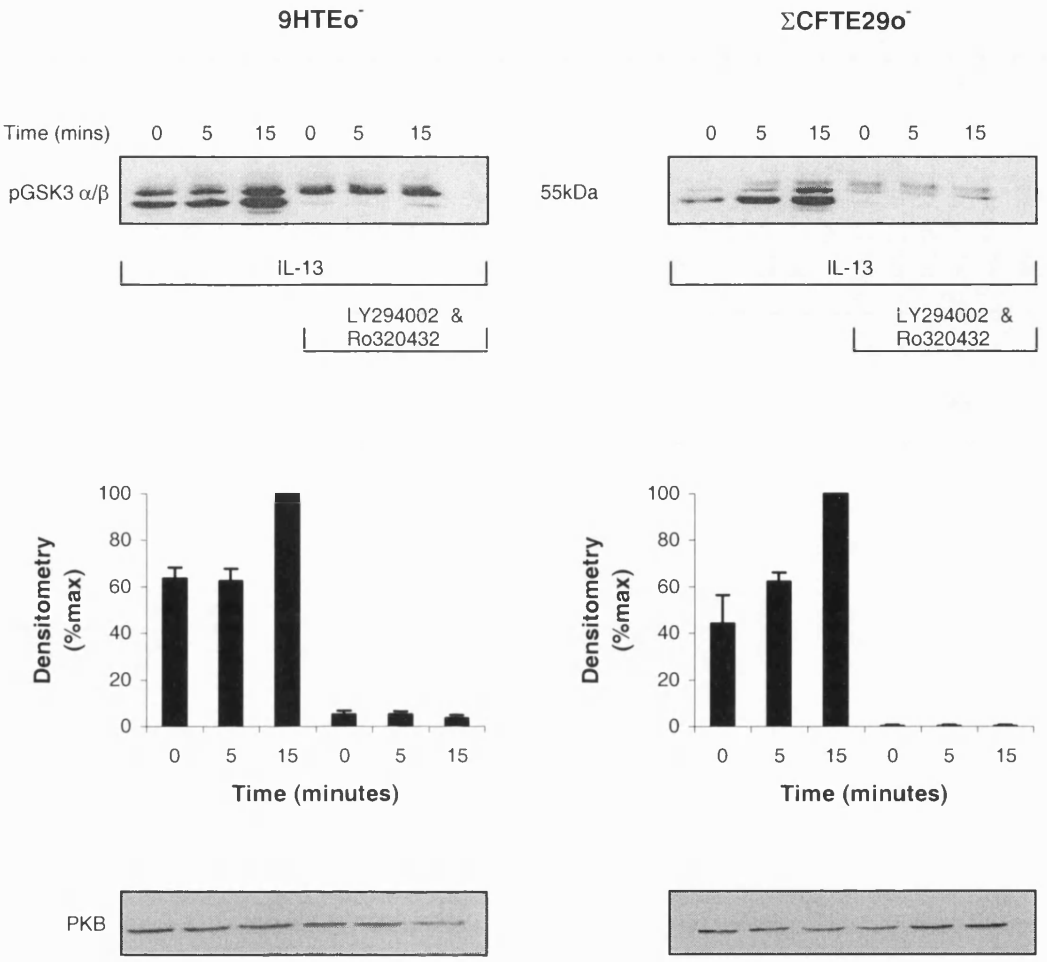


Figure 56 Phosphorylation of GSK3 in 9HTEo⁻ and ΣCFTE29o⁻ cells after stimulation with IL-13 (30 ng/ml) over a 15 minute time period at 37°C, +/- 1 hour pre-treatment with the PI3K inhibitor LY294002 (10 μM) and the PKC inhibitor Ro320432 (10 μM). The cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against phospho-(p) GSK3 (each blot is representative of 3 experiments), the middle panel is the densitometry analysis of the immunoblot (each bar is the mean ± SEM of 3 experiments) and the lower panel shows membranes probed with an antibody against pan PKB to check for equal loading.

As expected, when PD098059 (10 μM), SP600125 (10 μM) and Ro320432 (10 μM) were all used in combination, the levels of phosphorylated GSK3 were considerably reduced in both cell lines (Fig. 57). At the 20 minute time point, IL-13 induced phosphorylated GSK3 levels were reduced to 10% of maximum in the 9HTEo$^-$ cell line and to 1% of maximum in the ΣCFTE29o$^-$ cell line. It was probable that the combination of two or more inhibitors was having wide spread effects on the cells and the observed reduction in phosphorylated GSK3 levels was not solely due to the inhibition of particular GSK3 phosphorylating pathways. In order to eliminate this possibility, the combined effect of PD098059 (10 μM), SP600125 (10 μM) and Ro320432 (10 μM) on PKB phosphorylation was examined. When used alone none of these inhibitors had any effect on IL-13 induced phosphorylated PKB levels. Figure 58 demonstrates that this was also the case when these inhibitors were used together.

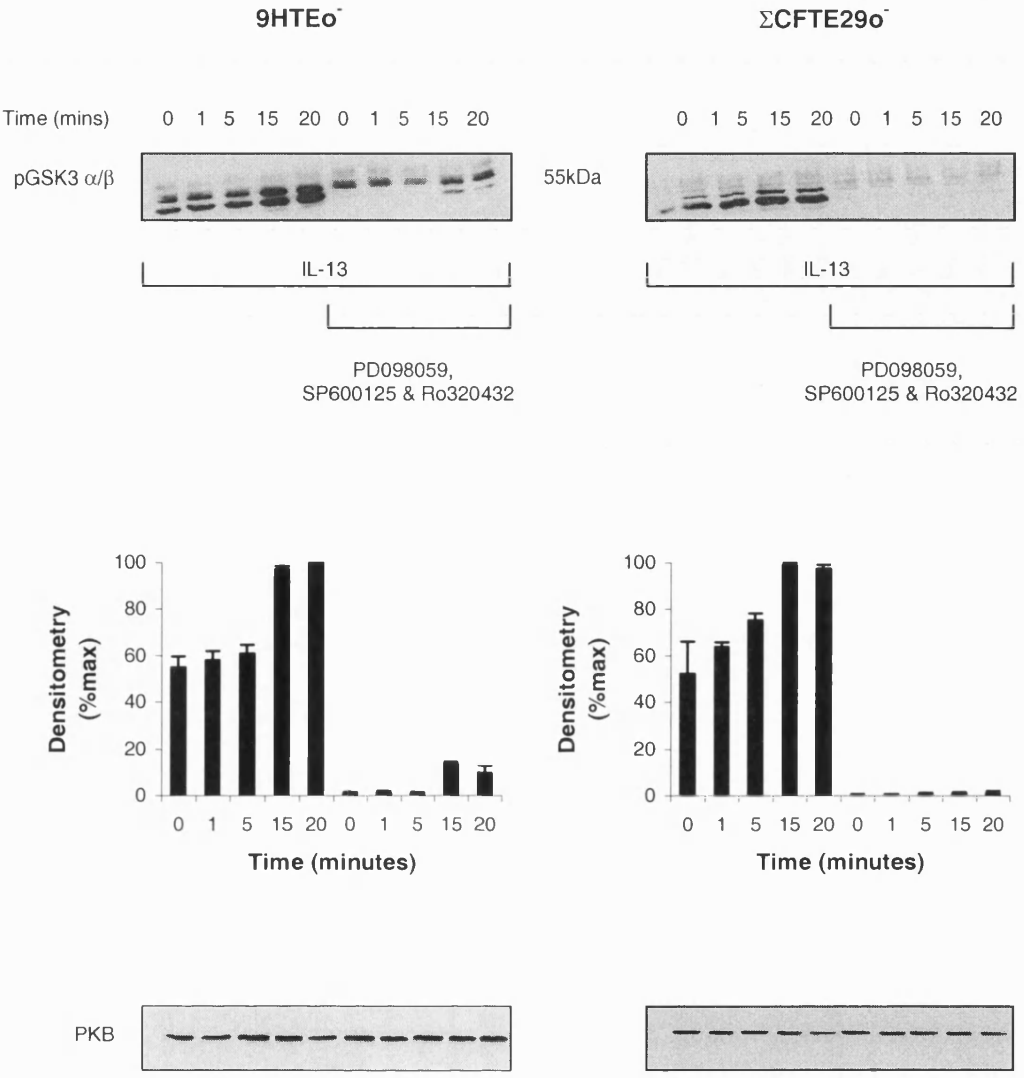


Figure 57 Phosphorylation of GSK3 in 9HTEo⁻ and ΣCFTE29o⁻ cells after stimulation with IL-13 (30 ng/ml) over a 15 minute time period at 37°C, +/- 1 hour pre-treatment with the ERK1/2 inhibitor PD098059 (10 μM), the JNK inhibitor SP600125 (10 μM) and the PKC inhibitor Ro320432 (10 μM). The cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against phospho-(p) GSK3 (each blot is representative of 3 experiments), the middle panel is the densitometry analysis of the immunoblot (each bar is the mean ± SEM of 3 experiments) and the lower panel shows membranes probed with an antibody against pan PKB to check for equal loading.

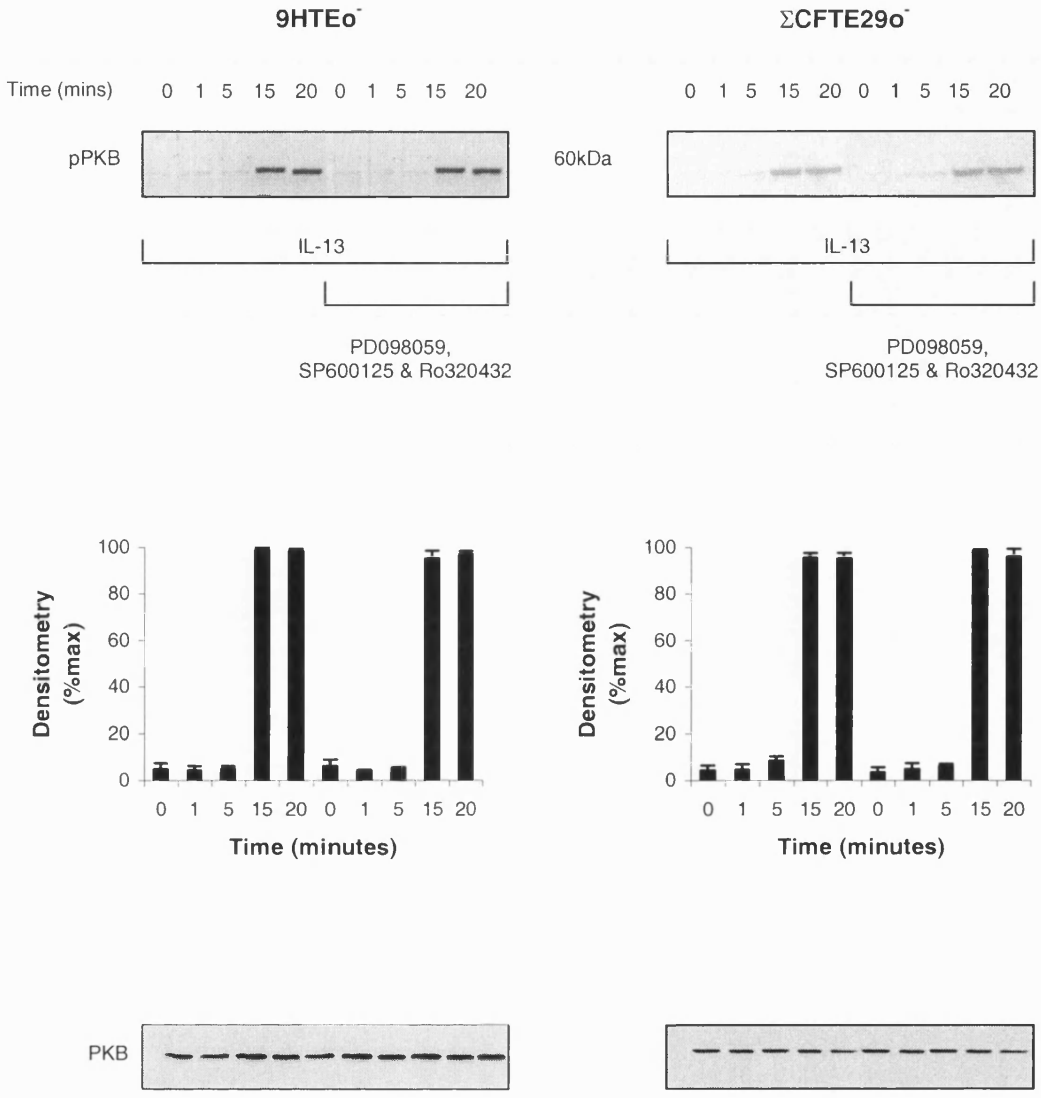


Figure 58 Phosphorylation of PKB in 9HTEo- and ΣCFTE29o- cells after stimulation with IL-13 (30 ng/ml) over a 15 minute time period at 37°C, +/- 1 hour pre-treatment with the ERK1/2 inhibitor PD098059 (10 μM), the JNK inhibitor SP600125 (10 μM) and the PKC inhibitor Ro320432 (10 μM). The cells were serum starved for 4 hours prior to treatment. The top panel is the immunoblot probed with a specific antibody against phospho-(p) PKB (each blot is representative of 3 experiments), the middle panel is the densitometry analysis of the immunoblot (each bar is the mean ± SEM of 3 experiments) and the lower panel shows membranes probed with an antibody against pan PKB to check for equal loading.

During the course of the experiments some interesting differences in the ratio of GSK3α/β were noted between the two cell lines. These ratios are summarised in table 3. In the absence of any inhibitors, GSK3α/β ratios of 0.6 ± 0.04 and 0.4 ± 0.02 were observed for unstimulated 9HTEo$^-$ and ΣCFTE29o$^-$ cells respectively. After 20 minutes stimulation with IL-13 (30 ng/ml) these ratios increased to 0.8 ± 0.02 and 0.6 ± 0.05 respectively. The change in these ratios was due to a greater increase in the levels of phosphorylated GSK3α compared to GSK3β in each case. The inclusion of certain inhibitors in the stimulations lead to larger differences in these ratios. For example, pre-treatment of 9HTEo$^-$ cells with the PI3K inhibitor LY294002 (10 μM) resulted in a GSK3α/β ratio of 1.0 ± 0.08 under basal conditions compared to 0.2 ± 0.06 for similarly treated ΣCFTE29o$^-$ cells. After 20 minutes stimulation with IL-13 (30 ng/ml) these ratios changed to 0.8 ± 0.02 for 9HTEo$^-$ cells and 0.3 ± 0.03 for ΣCFTE29o$^-$ cells. With the 9HTEo$^-$ cell line LY294002 seemed to inhibit both GSK3α and β to a similar degree, whereas with ΣCFTE29o$^-$ cells, the PI3K inhibitor appeared to have a greater effect on the α isoform than the β isoform. This differing action of LY294002 on GSK3α/β levels lead to the observed differences in α/β ratios between the cell lines. The JNK inhibitor SP600125 (10 μM) had a similar effect on GSK3α/β levels and also produced a large difference in GSK3α/β ratios, with values of 0.9 ± 0.01 for IL-13 stimulated 9HTEo$^-$ cells and 0.1 ± 0.04 for IL-13 stimulated ΣCFTE29o$^-$ cells. A similar pattern was observed when cells were pre-treated with the PKC inhibitor Ro320432 (10 μM) (ratios of 1.7 ± 0.15 for IL-13 stimulated 9HTEo$^-$ cells and 1.1 ± 0.20 for IL-13 stimulated ΣCFTE29o$^-$ cells). However, it was the combination of inhibitors that produced the greatest differences in GSK3α/β ratios. For instance, pre-treatment with LY294002 (10 μM) and the ERK1/2 inhibitor PD098059 (10 μM) resulted in a ratio of 0.8 ± 0.05 for IL-13

stimulated 9HTEo$^-$ cells and 4.3 ± 1.86 for IL-13 stimulated ΣCFTE29o$^-$ cells. In this case the high GSK3α/β ratio observed with the ΣCFTE29o$^-$ cell line seemed to result from the almost total abrogation of GSK3β by this particular inhibitor combination. Finally, when PD098059 (10 μM), SP600125 (10 μM) and Ro320432 (10 μM) were all used in combination GSK3α/β ratios of 7.6 ± 1.55 and 2.7 ± 1.02 were obtained for IL-13 stimulated 9HTEo$^-$ cells and ΣCFTE29o$^-$ cells respectively. The use of these three inhibitors resulted in a high level of GSK3β inhibition in both cell lines, whereas GSK3α was inhibited to a much lesser extent in the 9HTEo$^-$ cell line compared with the ΣCFTE29o$^-$ cell line.

Treatment		9HTEo⁻ GSK3 α/β ratio	ΣCFTE29o⁻ GSK3 α/β ratio
No inhibitor	basal	0.6 ± 0.04	0.4 ± 0.02
	20 min IL-13 (30 ng/ml)	0.8 ± 0.02	0.6 ± 0.05
+ LY294002 (10 μM)	basal	1.0 ± 0.08	0.2 ± 0.06
	20 min IL-13 (30 ng/ml)	0.8 ± 0.02	0.3 ± 0.03
+ PD098059 (10 μM)	basal	0.3 ± 0.07	0.2 ± 0.06
	20 min IL-13 (30 ng/ml)	0.7 ± 0.07	0.4 ± 0.02
+ SP600125 (10 μM)	basal	0.5 ± 0.06	0.1 ± 0.07
	20 min IL-13 (30 ng/ml)	0.9 ± 0.01	0.1 ± 0.04
+ Ro320432 (10 μM)	basal	1.3 ± 0.23	1.0 ± 0.51
	20 min IL-13 (30 ng/ml)	1.7 ± 0.15	1.1 ± 0.20
+ LY294002 (10 μM) & PD098059 (10 μM)			
	basal	1.3 ± 0.37	2.0 ± 1.50
	20 min IL-13 (30 ng/ml)	0.8 ± 0.05	4.3 ± 1.86
+ LY294002 (10 μM) & SP600125 (10 μM)			
	basal	0.8 ± 0.27	0.1 ± 0.06
	20 min IL-13 (30 ng/ml)	0.4 ± 0.10	0.3 ± 0.04
+ LY294002 (10 μM) & Ro320432 (10 μM)			
	basal	10.8 ± 3.48	12.0 ± 4.17
	20 min IL-13 (30 ng/ml)	24.7 ± 6.07	22.0 ± 9.88
+ PD098059 (10 μM), SP600125 (10 μM) & Ro320432 (10 μM)	basal	53.0 ± 19.16	7.6 ± 2.65
	20 min IL-13 (30 ng/ml)	7.6 ± 1.55	2.7 ± 1.02

Table 3 Summary of GSK3 α/β ratios in 9HTEo⁻ and ΣCFTE29o⁻ cells under basal conditions and after 20 minutes stimulation with IL-13 (30ng/ml) +/- 1 hour pre-treatment with various combinations of the PI3K inhibitor LY294002 (10 μM), the ERK1/2 inhibitor PD098059 (10 μM), the JNK inhibitor SP600125 (10 μM) and the PKC inhibitor Ro320432 (10 μM). Each value is the mean ± SEM of three experiments.

5.3 PHOSPHATIDYLINOSITOL 3-KINASE ACTIVITY IN 9HTEo$^-$ AND ΣCFTE29o$^-$ CELLS

Confluent monolayers of 9HTEo$^-$ and ΣCFTE29o$^-$ cells were serum starved for 4 hours, resuspended in FCS-free medium and then pre-incubated with wortmannin (100 nM) or vehicle control for 15 minutes at 37°C. The cells were subsequently stimulated at 37°C with vehicle or IL-13 (30 ng/ml) for 1-15 minutes. PI3K was immunoprecipitated from cell lysates and a lipid kinase assay was carried out as described in the materials and methods section. Figure 59 shows the result of one such lipid kinase assay. Basal levels of PtdIns-3-P appeared to be low in both the unstimulated 9HTEo$^-$ and ΣCFTE29o$^-$ cell samples (7% of maximum and 8% of maximum respectively), suggesting relatively low basal levels of PI3K activity (or possibly high basal phospatase activity). After 1 minute IL-13 stimulation the levels of PtdIns-3-P had begun to increase and had peaked by 5 minutes in both cell lines (70% of maximum for the 9HTEo$^-$ cell line and 100% for the ΣCFTE29o$^-$ cell line). After 15 minutes IL-13 stimulation, PtdIns-3-P levels were still higher than basal but had started to decrease in both cell lines (down to 25% of maximum in 9HTEo$^-$ cells and 85% of maximum in ΣCFTE29o- cells). Pretreatment of the cells with the PI3K inhibitor wortmannin (100 nM) reduced maximum PtdIns-3-P levels almost to basal in both the 9HTEo$^-$ and ΣCFTE29o$^-$ cell samples (10% of maximum and 15% of maximum respectively).

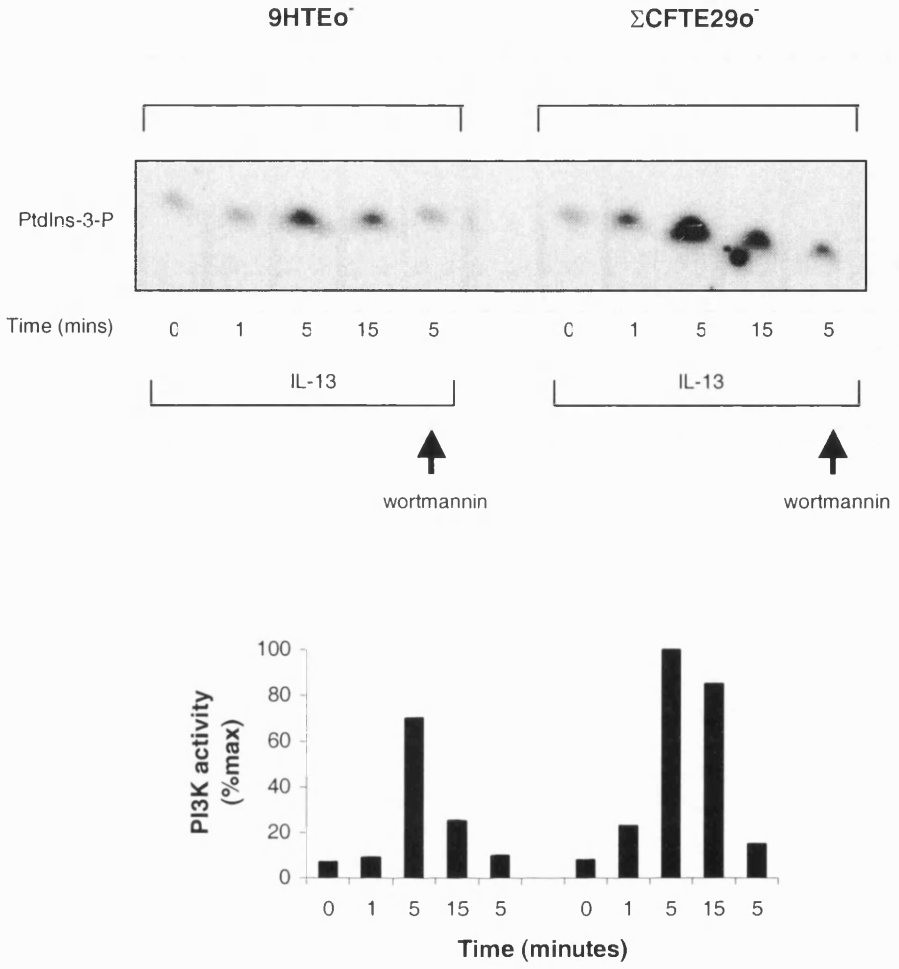


Figure 59 ***In vitro*** **lipid kinase activity of immunoprecipitated PI3K from 9HTEo⁻ and ΣCFTE29o⁻ cell lysates after stimulation with IL-13 (30 ng/ml) over a 15 minute time period at 37°C, +/- 15 minutes pre-treatment with the PI3K inhibitor wortmannin (100 nM).** The immunoprecipitates were washed and analysed for PI3K activity using PtdIns as a substrate. Extraction and TLC separation of the lipid products were performed as described in the materials and methods section. Lipids were detected by exposure to film at –70°C. The cells were serum starved for 4 hours prior to treatment. The top panel is the autoradiograph and the lower panel is the densitometry analysis of the autoradiograph. The data are representative of 2 experiments.

5.4 ACTIVITY OF THE LIPID PHOSPHATASE PTEN IN 9HTEo$^-$ AND ΣCFTE29o$^-$ CELLS

Confluent monolayers of 9HTEo$^-$ and ΣCFTE29o$^-$ cells were serum starved for 4 hours and the levels of PTEN in unstimulated cell lysates were determined via immunoblot analysis as described in the materials and methods section. PTEN was found to be present in both 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines (Fig 60). However, immunoblot analysis of PTEN did not provide any indication of the activity of this lipid phosphatase. In order to establish basal phsophatase activity in the two cell lines, confluent monolayers of 9HTEo$^-$ and ΣCFTE29o$^-$ cells were serum starved for 4 hours and PTEN was immunoprecipitated from cell lysates. A lipid phosphatase assay was then carried out as described in the materials and methods section. The lipids generated during the assay were separated by high-pressure liquid chromatography (HPLC) and detected via scintillation counting. Figure 61 shows the result of one such phosphatase assay. The substrate for PTEN, [^{3}H]-PtdIns-1,3,4,5-P_4 eluted from the HPLC column after the reaction product, [^{3}H]-PtdIns-1,4,5-P_3. By calculating the substrate : product ratio for each cell line, it was possible to make comparisons of basal PTEN activity.

9HTEo$^-$	: substrate	: product	([^{3}H]-PtdIns-1,3,4,5-P_4 : [^{3}H]-PtdIns-1,4,5-P_3)
	169.60	: 120.71	
	1.4	: 1	

ΣCFTE29o$^-$: substrate : product ([^{3}H]-PtdIns-1,3,4,5-P_4 : [^{3}H]-PtdIns-1,4,5-P_3)

183.64 : 97.77

1.8 : 1

The difference in substrate : product ratio between the two cell lines was very small and suggests that there was no major difference in basal PTEN activity between the 9HTEo$^-$ and ΣCFTE29o$^-$ cell samples.

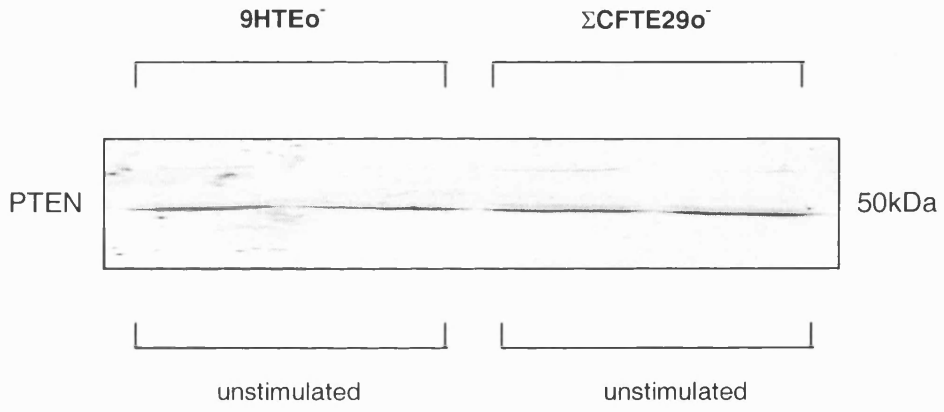


Figure 60 Immunoblot analysis of unstimulated 9HTEo$^-$ and ΣCFTE29o$^-$ cell lysates probed with a specific antibody against the lipid phosphatase PTEN. The cells were serum starved for 4 hours prior to lysis. The data are representative of 2 experiments.

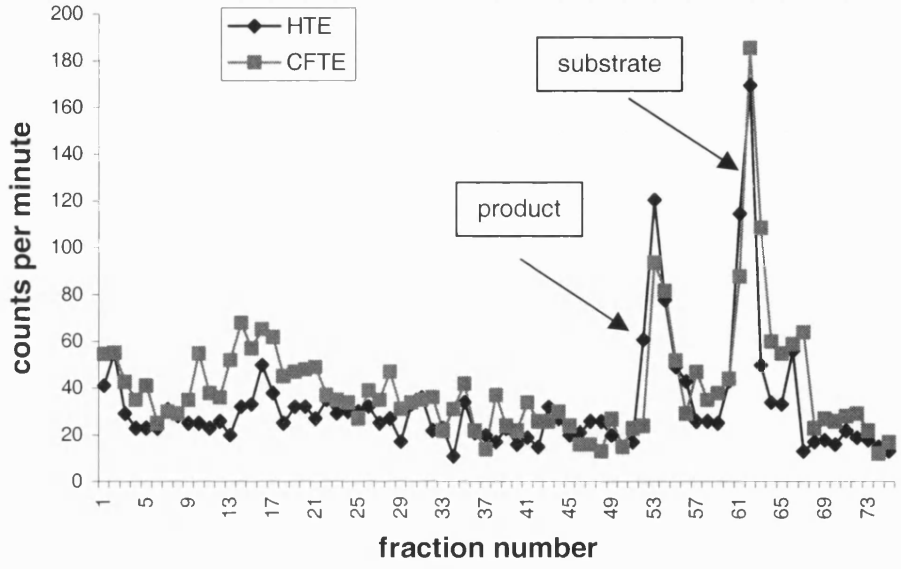


Figure 61 ***In vitro*** **lipid phosphatase activity of immunoprecipitated PTEN from unstimulated 9HTEo$^-$ and ΣCFTE29o$^-$ cell lysates.** The immunoprecipitates were washed and analysed for PTEN phosphatase activity using [^{3}H]-PtdIns-1,3,4,5-P_4 as a substrate. Extraction and HPLC separation of the lipid products were performed as described in the materials and methods section. Lipids were detected via scintillation counting. The cells were serum starved for 4 hours prior to lysis. The data are representative of 2 experiments.

5.5 DISCUSSION

The D-3 phosphoinositide lipids have been linked to the triggering of a diverse array of cellular responses including cell survival, mitogenesis, membrane trafficking, glucose transport, neurite outgrowth, membrane ruffling, superoxide production, actin polymerisation and chemotaxis (Wright & Ward, 2001). Recently, a number of proteins have been identified that bind directly to these D-3 phosphoinositide lipids, including protein kinase B (PKB) and phosphoinositide-dependent kinase-1 (PDK1) (Rameh & Cantley, 1999; Stephens *et al.*, 1998). Activated PKB has been implicated in the activation of the transcription factor NF-κB in human leucocytes and a variety of human cancer cell lines (Ozes *et al.*, 1999; Romashkova & Makarov, 1999; Kane *et al.*, 1999; Madrid *et al.*, 2000). Furthermore, glycogen synthase kinase-3 (GSK3), a major downstream target of PKB, has been linked to the regulation of a range of transcription factors including c-Jun (part of the AP-1 transcription factor complex), cyclic-AMP response element binding protein (CREB) and c-Myc (Woodgett, 1991; DeGroot *et al.*, 1992; Plyte *et al.*, 1992; Fiol *et al.*, 1994). The regulation of inflammatory genes, for instance those for iNOS and IL-8, have been linked to the activation of transcription factors such as NF-κB and AP-1 (Lowenstein *et al.*, 1993; Mukaida *et al.*, 1990; Kunsch & Rosen, 1993; Matsusaka *et al.*, 1993; Oliveira *et al.*, 1994) and PI3K/PKB/GSK3 signalling may well be involved in these regulatory processes. Recently PKB and GSK3 have also been shown to play an important role in the regulation of cell survival mechanisms (Coffer *et al.*, 1998; Pap & Cooper, 1998). There is a large body of evidence suggesting that iNOS and IL-8 production are dysregulated in CF airway epithelia (Light *et al.*, 1989; Grasemann *et al.*, 1997; Kelley & Drumm, 1998; Ruef *et al.*, 1993; Stecenko *et al.*, 1997; Black *et al.*, 1998; Schwiebert *et al.*, 1999; Massengale

et al., 1999) and it has also been suggested that cell survival mechanisms may be altered in CF (Maiuri, 1997). In the light of these findings it seemed reasonable to examine PI3K/PKB/GSK3 signalling in airway epithelium and to see if any differences existed in this signalling pathway between CF and non-CF cells.

In the gut epithelial cell line HT-29, the Th2 cytokine IL-13 has been shown to induce a concentration and time-dependent increase in the formation of the lipid products of PI3K activity, namely PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 and this has been linked to the inhibition of cytokine-induced iNOS expression (Wright *et al.*, 1997). Stimulation of 9HTEo$^-$ and ΣCFTE29o$^-$ cells with IL-13 (30 ng/ml) resulted in phosphorylation of both PKB and GSK3α/β within a 15 minute time period and these levels were comparable between the two cell lines. The phosphorylation of PKB was shown to be dependent on PI3K activity. PI3K inhibition also partially reduced GSK3 phosphorylation. This link between growth factor stimulation, PI3K activation and PKB/GSK3 phosphorylation had previously been demonstrated in a range of other systems (Coffer *et al.*, 1998; Frame & Cohen, 2001) but until now had not been confirmed in airway epithelium. Interestingly, no consistent differences in basal PKB and GSK3 phosphorylation levels were observed between the 9HTEo$^-$ and ΣCFTE29o$^-$ cells and therefore it appears that both basal and IL-13 stimulated PI3K activity (as measured by PKB and GSK3 phosphorylation) were similar in these cell lines.

PKB is the most extensively studied target of PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 and its phosphorylation is thought to be regulated exclusively by phosphoinositide-dependent kinases like PDK1 (Franke *et al.*, 1997; Downward *et al.*, 1998). The results of this study support such a proposal, as PKB phosphorylation in the 9HTEo$^-$ and ΣCFTE29o$^-$

airway epithelial cell lines was shown to be independent of any MAPK activity (ERK1/2, p38 and JNK independent) and PKC independent. On the other hand, it has been demonstrated *in vitro* that MAPK pathways can phosphorylate GSK3 on the same N-terminal serine residues that are phosphorylated *in vivo* by PKB (Sutherland *et al.*, 1993). It has also been established that the mammalian target of rapamycin (mTOR) signalling pathway can lead to GSK3 phosphorylation and inactivation in some systems (Frame & Cohen, 2001). The results from this study have established that certain MAPK pathways and the PKC signalling pathway are involved in GSK3 phosphorylation in airway epithelial cells. As phosphorylation by GSK3 appears to inhibit the activities of proteins involved in a range of biosynthetic processes, it makes sense that GSK3 should be inactivated through a variety of means. Such regulation may provide a coordinated mechanism for activating a spectrum of anabolic pathways in response to particular stimuli. It is also possible that individual growth factors inhibit GSK3 via more than one pathway. IL-13 may have acted in such a way in the airway epithelial cells used in this study, as PI3K, MAPK and PKC inhibitors all attenuated IL-13 stimulated GSK3 phosphorylation.

Although the MAPK and PKC signalling pathways were shown to be involved in phosphorylating GSK3 in both the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines, some interesting differences were observed in the relative contributions of these pathways to GSK3 inactivation. Both the ERK1/2 and JNK MAPK pathways appeared to have a greater role in GSK3 phosphorylation in ΣCFTE29o$^-$ cells than in 9HTEo$^-$ cells. However, the p38 branch of the MAPK pathway was not found to be involved in GSK3 phosphorylation in either cell line. Finally, the PKC signalling pathway was also shown to play a larger role in GSK3 inactivation in the ΣCFTE29o$^-$ cell line compared to the

9HTEo$^-$ cell line. These data suggest that GSK3 phosphorylation is subject to different regulatory mechanisms in 9HTEo$^-$ and ΣCFTE29o$^-$ cells. This finding could have implications for the control of the many cellular processes that are regulated by GSK3, such as transcription factor activation and the regulation of cell survival pathways.

It is important to note that it was the β isoform of GSK3 that was analysed when comparing phosphorylated GSK3 levels between the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines. This isoform has been implicated in the regulation of gene transcription and cell survival (Woodgett, 1991; DeGroot *et al.*, 1992; Plyte *et al.*, 1992; Fiol *et al.*, 1994), while little is known about the signalling properties of GSK3α. Interestingly, inhibition of ERK1/2, JNK and PKC all substantially decreased the levels of phosphorylated GSK3α in the ΣCFTE29o$^-$ cell line whilst having little effect on GSK3α levels in the 9HTEo$^-$ cell line. In addition, some interesting differences in certain GSK3α/β ratios were observed between the two cell lines. However, until the role of GSK3α in the regulation of cellular activities is elucidated further, the relevance of these particular findings will remain unclear.

The results from the *in vitro* lipid kinase assays gave some support to the data from the immunoblot experiments. In these assays, the levels of PtdIns-3-P visualised on the autoradiograph corresponded directly to PI3K activity. No differences were observed in the basal levels of PI3K activity in the two cell lines. This corresponded with the levels of phosphorylated PKB and GSK3 found in these cells in the absence of cytokine stimulation. Treatment of both cell lines with IL-13 (30 ng/ml) resulted in PI3K activation, which peaked after 5 minutes and was attenuated by the PI3K inhibitor wortmannin (100 nM). It is interesting to note that after 15 minutes IL-13 stimulation, PI3K activity began to decrease in the 9HTEo$^-$ and ΣCFTE29o$^-$ cells whereas

phosphorylated PKB levels showed no signs of decreasing 90 minutes after IL-13 stimulation. It could be argued that phosphorylated PKB levels remained high after this length of time due to the constant presence of IL-13 in the cell culture medium. However, IL-13 was also constantly present in the cell culture medium of cells used for the *in vitro* lipid kinase assays. It would seem therefore that a short burst of PI3K activity is associated with prolonged PKB phosphorylation. On the other hand, although PI3K activity started to decrease 15 minutes after IL-13 stimulation in both cell lines, it had not returned to basal levels. Perhaps this level of PI3K activity was sufficient to maintain the long-lasting phosphorylation of PKB. It is apparent that IL-13 stimulations need to be carried out over longer time periods in order to establish the true relationship between PI3K activity and phosphorylated PKB levels in these airway epithelial cells.

Wortmannin was used instead of LY294002 to inhibit PI3K activity during the *in vitro* lipid kinase assays. Both these compounds are effective inhibitors of PI3K but differ in their modes of action. Wortmannin is classified as a non-competitive inhibitor of PI3K and is effective at nanomolar concentrations (Wymann *et al.*, 1996), while LY294002 is classed as a competitive inhibitor of PI3K and is effective at micromolar concentrations (Vlahos *et al.*, 1994). Wortmannin is also extremely light sensitive and degrades rapidly, whereas LY294002 is much more stable (Wymann *et al.*, 1996). Due to the inherent instability of wortmannin, LY294002 was used in all the immunoblot stimulations. However, the fact that LY294002 is a competitive inhibitor of PI3K meant that it was unsuitable for use with the *in vitro* lipid kinase assays. It is highly unlikely that this inhibitor would have remained in association with PI3K during the assay procedure due to the high levels of PtdIns present in the assay reaction mixture.

The *in vitro* lipid kinase assays also helped to shed some light on the nature of the PI3K isoforms present in the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines. Unstimulated and IL-13 stimulated cell lysates were incubated overnight with mouse monoclonal anti-PI3K p85 antibody. This antibody only recognises PI3K isoforms that consist of a p85 regulatory subunit. Only class 1_A PI3Ks have this particular type of regulatory subunit, which is complexed to a p110 catalytic subunit (Vanhaesebroeck *et al.*, 1997). The fact that PI3K activity was readily demonstrated after p85 immunoprecipitation suggests that 9HTEo$^-$ and ΣCFTE29o$^-$ airway epithelial cells contain at least one class 1_A PI3K isoform. Of course, other PI3K isoforms could also be present in these cells. It may be that the prolonged phosphorylation of PKB in response to IL-13 stimulation was the result of the activity of PI3K isoforms other than the immunoprecipitated p85/p110 class 1_A isoform. Further work will need to be carried out in order to identify these isoforms and establish their relative activities.

It is well established that phosphoinositide second messengers are essential components of signalling pathways that control critical cellular functions. Consequently, it is not surprising that the regulation of cellular phosphoinositide levels is a complex process involving the interplay of various phosphoinositide kinases as well as phosphatases and lipases. Together these enzymes control the biosynthesis and degradation of inositol lipids. The phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10), also known as MMAC1 (mutated in multiple advanced cancers 1) and TEP1 (transforming growth factor β-regulated and epithelial-cell-enriched phosphatase), has both protein and lipid phosphatase activity and is a regulator of the phosphoinositide second messenger PtdIns-3,4,5-P_3 in many cell types (Krystal, 2000). The *PTEN* gene was originally identified as a human tumour

suppressor gene located on chromosome 10q23.3, a region associated with high grade glioblastomas, prostate cancer and breast cancer (Li *et al.*, 1997; Li & Sun, 1997; Steck *et al.*, 1997). However, more recently PTEN has been shown to downregulate signalling through PKB by dephosphorylating PtdIns-3,4,5-P_3 at the 3'-OH position. This action prevents the activation of PDK1 and PKB and therefore inhibits cell proliferation and anti-apoptotic signalling (Stambolic *et al.*, 1998; Cantley & Neel, 1999; Maehama & Dixon, 1999; DiCristofano *et al.*, 1999).

This study hypothesises that aberrant PI3K signalling may be responsible for some of the lung manifestations observed in CF. If this is the case it could be related to a deficient PTEN regulatory system. The fact that basal levels of PI3K activity and phosphorylated PKB were low in both cell lines and were similar in IL-13 stimulated cells suggested that this was not the case. The PTEN immunoblot analysis verified that this particular lipid phosphatase was present in the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines and the results from the *in vitro* lipid phosphatase assays suggested that there was little difference in PTEN activity between these two cell lines in the absence of cytokine stimulation. It will be necessary to establish the activity of PTEN in IL-13 stimulated cells in order to say with any certainty whether or not there are deficiencies in PTEN regulation between the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines.

In summary it can be said that there are no differences in PI3K activity between 9HTEo$^-$ and ΣCFTE29o$^-$ cells and no differences in basal PTEN activity. However, there are

some interesting differences in the regulation of the PI3K downstream effector GSK3α/β, which may impact on a range of cellular processes.

5.6 SUMMARY OF RESULTS

- Basal levels of phosphorylated PKB were low in both 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines, but increased considerably after 15 minutes stimulation with IL-13 (30 ng/ml)
- The PI3K inhibitor LY294002 (10 μM) completely inhibited IL-13 stimulated PKB phosphorylation in both cell lines.
- Levels of phosphorylated PKB were still high 90 minutes after IL-13 stimulation in both cell lines.
- The MAPK inhibitors (PD098059, 10 μM; SB203580, 10 μM; SP600125, 10 μM) and the PKC inhibitor (Ro320432, 10 μM) either alone or in combiniation had no effect on IL-13 stimulated PKB phosphorylation in either cell line.
- The ERK1/2 inhibitor PD098959 (10 μM), the JNK inhibitor SB203580 (10 μM) and the PKC inhibitor Ro320432 (10 μM) all had a greater inhibitory effect on IL-13 stimulated phosphorylated GSK3 levels in ΣCFTE29o$^-$ cells compared with 9HTEo$^-$ cells when used alone or in combination with LY294002 (10 μM).
- The p38 inhibitor SB203580 (10 μM) had no effect on phosphorylated GSK3 levels in either cell line.
- Differences in GSK3α/β ratios were observed between the cell lines after inhibitor treatment and IL-13 stimulation.
- Basal levels of PtdIns-3-P were low in both cell lines and peaked after 5 minutes IL-13 stimulation.
- IL-13 stimulated PtdIns-3-P accumulation was inhibited by wortmannin (100 nM) in both cell lines.
- Basal PTEN activity was low in both cell lines.

6 DISCUSSION

The distinguishing feature of CF lung disease is the inability of the host to clear the initial colonisation and subsequent infection of the airways by *Staphyloccocus aureus*, *Pseudomonas aeruginosa* and sometimes, *Burkholderia cepacia*. This is despite an intense inflammatory response that is characterised by an influx of neutrophils and macrophages and the secretion of proinflammatory cytokines such as IL-1β, TNFα and IFNγ. Activated inflammatory cells also release proteases, oxidants and defensins, which exacerbate the inflammatory response. The overall result is obstructive lung disease and eventual tissue destruction. It has been proposed that a tendency towards excessive inflammation is intrinsic in CF (Cantin, 1995) and this has been linked to the finding that CF airway epithelial cell lines produce higher levels of the neutrophil attracting chemokine IL-8, in response to *Pseudomonas* stimulation, than non-CF airway epithelial cell lines (DiMango *et al.*, 1995; Bryan *et al.*, 1998; Tabary *et al.*, 1999). NO levels have also been shown to be aberrant in CF airways. It has been demonstrated that NO levels in the exhaled air from CF patients are reduced compared with normal controls (Light *et al.*, 1989; Grasemann *et al.*, 1997) and this has been correlated with the observation that iNOS expression is reduced in murine CF airway epithelium and the human CF epithelial cell line CFBE41o$^-$ (Kelley & Drumm, 1998; Meng *et al.*, 1998). The induction of NO in many animal models is directly associated with the ability of the host to contain microbial proliferation (Stenger *et al.*, 1996; MacMicking *et al.*, 1997). Therefore, if iNOS expression is reduced in human airway epithelium it might help to explain why the human CF lung is so susceptible to bacterial colonisation. In view of these findings, this study set out to examine the role of well characterised human airway epithelial cell lines in the production of IL-8 and NO, in the

context of an inflammatory situation, and to define which agents might affect their regulation.

The airway epithelial cell lines 9HTEo$^-$, ΣCFTE29o$^-$, 9HTEo$^-$/pCEP#2 and 9HTEo$^-$ /pCEPRF all produced a small amount of NO (as determined by nitrite release) in the absence of cytokine stimulation, but this was not found to be the result of constitutive iNOS activity. Curiously, the CF phenotype cells produced significantly more nitrite than their non-CF counterparts and none of the cell lines produced increased nitrite in response to stimulation with proinflammatory cytokines. These cell lines also produced small amounts of IL-8 in the absence of cytokine stimulation and these levels were significantly increased by stimulation of the cells with IL-1β alone, TNFα alone or IFNγ, IL-1β and TNFα in combination. It was interesting to note that while the cytokine stimulated IL-8 levels produced by the 9HTEo$^-$ cell line were significantly higher than those produced by the ΣCFTE29o$^-$ cell line, the converse was true for the control and CF-phenotype pCEP cell lines.

It appears that the proinflammatory cytokine requirement for the induction of iNOS in immortalised airway epithelial cells varies depending upon the cell line being studied (Asano *et al.*, 1994; Robbins *et al.*, 1994; Guo *et al.*, 1997; Watkins *et al.*, 1997; Uetani *et al.*, 2001) and may even be a consequence of how the cell lines were created. In a number of airway epithelial cell lines iNOS expression cannot be induced in response to proinflammatory cytokine stimulation (Guo *et al.*, 1997; Uetani *et al.*, 2001). This also appears to be the case with the 9HTEo$^-$, ΣCFTE29o$^-$, 9HTEo$^-$/pCEP#2 and 9HTEo$^-$ /pCEPRF cell lines used in this study. The induction of iNOS, in response to cytokine stimulation, has been demonstrated in the type II alveolar carcinoma cell line, A549

(Asano *et al.*, 1994; Robbins *et al.*, 1994) and by some researchers in the bronchial epithelial cell line, BEAS-2B (Watkins *et al.*, 1997). However, it should be noted that in the latter study the authors observed that cytokine stimulated iNOS mRNA expression diminished rapidly with successive cell passages. Interestingly, a recent study by Donnelly & Barnes (2002) reported that the A549 and BEAS-2B cell lines produced very little nitrite, under both basal and stimulated conditions, compared with primary cells.

The induction of iNOS gene expression seems to be regulated differently between various human cell lines (Charles *et al.*, 1993; Geller *et al.*, 1993; Linn *et al.*, 1997) and such differences in regulation may explain the inconsistencies observed in the production of NO between different human airway epithelial cell lines. Although numerous intracellular signal transduction pathways have been implicated in iNOS gene expression, including those leading to NF-κB and AP-1 activation (DeVera *et al.*, 1996; Marks-Konczalik *et al.*, 1998), IFNγ activation of STAT-1 and IRF-1 is also likely to be essential for iNOS induction in human cells (Guo *et al.*, 2000; Uetani *et al.*, 2000). It has been demonstrated that infection of cells with adenovirus may impair STAT-1 activation (Look *et al.*, 1998) and this could explain why cell lines created by the transformation of airway epithelial cells with adenovirus-12 SV40 hybrid virus, such as the BEAS-2B cell line, show low levels of iNOS expression in response to cytokine stimulation. However, the IFNγ signalling pathway and subsequent IFNγ-inducible gene transcription are reportedly intact in BEAS-2B cells (Uetani *et al.*, 2001). Taken together with the knowledge that IFNγ potentiated IL-1β and TNFα induced IL-8 production in the 9HTEo$^-$, ΣCFTE29o$^-$, 9HTEo$^-$/pCEP#2 and 9HTEo$^-$/pCEPRF cells, at least some of the IFNγ signalling pathway is also intact in these cell lines. It is interesting to note that Uetani and colleagues (2001), were unable to show iNOS

expression in response to cytokine stimulation in BEAS-2B and BET-1A cells, although they did demonstrate that TNFα strongly induced NF-κB activation in these cell lines. Once again, the fact that cytokine stimulation of the 9HTEo$^-$, ΣCFTE29o$^-$, 9HTEo$^-$/pCEP#2 and 9HTEo$^-$/pCEPRF cell lines resulted in high levels of IL-8 production, confirms that the NF-κB activation pathway must also be intact in these particular cells. Uetani *et al.* (2001), were able to induce iNOS expression in BEAS-2B cells in response to stimulation with proinflammatory cytokines and double-stranded RNA (dsRNA). However, this combination had no effect on iNOS expression in the BET-1A cell line. Similarly in this study, the stimulation of 9HTEo$^-$, ΣCFTE29o$^-$, 9HTEo$^-$/pCEP#2 and 9HTEo$^-$/pCEPRF cells with cytokines and dsRNA did not lead to nitrite induction. It therefore appears that although the expression of iNOS in transformed human airway epithelial cells may well be dependent on the activation of STAT-1, IRF-1 and NF-κB, other pathways must also be involved and these may vary depending on the cell line in question. Taking all these studies and variations into consideration, it seems that immortalised airway epithelial cell lines may be poor models for the study of human airway epithelial iNOS expression.

As well as the reported inconsistencies in iNOS expression between various airway epithelial cell lines, it has also been demonstrated that primary cells that have been passaged once show considerably reduced levels of nitrite release, under both basal and cytokine stimulated conditions, compared with freshly isolated cells (Donnelly & Barnes, 2002). This finding is supported by the work of Guo and colleagues (1995) who demonstrated that freshly isolated human bronchial epithelial cells lose their ability to synthesise iNOS mRNA within 72 hours. These findings suggest that the control of iNOS expression in human airway epithelium is dependent on a combination of *in vivo*

factors or exposures, to which airway epithelial cells are uniquely responsive and support the premise that multiple signal transduction pathways are involved in the regulation of iNOS expression in human airway epithelium.

The lack of a suitable model for the study of NO regulation in human airway epithelium makes it very difficult to comment on the relevance of the observations that iNOS expression is reduced in the human CF epithelial cell line CFBE41o$^-$ and in murine CF airway epithelium (Kelley & Drumm, 1998; Meng *et al.*, 1998). In the study by Meng and colleagues (1998) the expression of iNOS mRNA in cytokine stimulated and unstimulated human CF (CFBE41o$^-$) and non-CF (16HBE14o$^-$) bronchial epithelial cell lines was detected by reverse transcriptase-polymerase chain reaction (RT-PCR). The authors demonstrated the induction of iNOS mRNA in cytokine stimulated 16HBE14o$^-$ cells, but in contrast no specific target mRNA band was detected in either cytokine stimulated or unstimulated CFBE41o$^-$ cells amplified for the same number of cycles. Meng *et al.* (1998) concluded that the absence of iNOS mRNA in the CFBE41o- cell line was due to a lack of iNOS gene expression, most likely resulting from abnormalities in the signalling system that normally causes induction. However, the authors only looked at RT-PCR amplified iNOS message and did not look directly at iNOS mRNA induction, iNOS protein expression or NO production. In the manner used RT-PCR is not a quantitative procedure and in the absence of any direct measurements of NO production or iNOS expression, it is difficult to establish the true magnitude or significance of the differences found. It is also unwise to extrapolate data regarding the regulation of murine iNOS to human tissue. Much of the work regarding the regulation of iNOS expression has been performed in murine models (Robbins *et al.*, 1994; Walker *et al.*, 1997; Jeon *et al.*, 1998). In these systems iNOS is readily induced and produces large quantities of NO. However, as demonstrated, studies of human iNOS have

proved to be less successful. There are differences in the promoters of human and murine iNOS that are thought to account for the hyporesponsiveness of human iNOS (DeVera *et al.*, 1996; Taylor *et al.*, 1998; Ganster *et al.*, 2001; Mellott *et al.*, 2001). In particular it is thought that NF-κB is more important in the regulation of murine iNOS (Chu *et al.*, 1998) whilst the IFNγ signalling pathway is more important in the regulation of the human gene (Bories *et al.*, 1999; Guo *et al.*, 1997). It would seem that human iNOS expression and hence NO production is more tightly regulated than murine models. This may be linked to the suggestion that in human tissue NO has a role to control the regulation of gene expression rather than just directing cytotoxicity (Kroncke *et al.*, 1998). In summary, it appears that until the conditions for iNOS expression are better characterised in both human primary airway epithelial cells and human airway epithelial cell lines, there will continue to be a need for a reliable model system to further investigate the complex mechanisms leading to iNOS gene expression in human airway epithelium.

The transcriptional control of IL-8 expression has been linked to the activation of a range of transcription factors, including IRF-1, AP-1, AP-2, C/EBP and NF-IL-6 (Mukaida *et al.*, 1990; Kunsch & Rosen, 1993; Matsusaka *et al.*, 1993; Oliveira *et al.*, 1994). However, activation of NF-κB is thought to be the most crucial step for the initiation of IL-8 gene transcription in the majority of cell types (Mukaida *et al.*, 1994). As mentioned earlier, the fact that the 9HTEo$^-$, ΣCFTE29o$^-$, 9HTEo$^-$/pCEP#2 and 9HTEo$^-$/pCEPRF cell lines used in this study all produced high levels of IL-8 in response to cytokine stimulation suggests that they contain intact NF-κB activation pathways. In addition, the synergistic affect of IFNγ on IL-1β and TNFα stimulated IL-8 production indicates that, in these particular cells, NF-κB may be cooperating with

elements of the IRF-1/STAT-1/IFNγ signalling pathway in order to achieve maximum IL-8 generation. It is possible that other transcription factors, such as AP-1, are also involved in the regulation of IL-8 production in these cell lines in response to proinflammatory cytokine stimulation.

The molecular mechanism involved in IL-8 gene transcription is thought to differ, even within the same cell, depending upon the stimulus used. For example AP-1 has been shown to be the preferred transcription factor (over NF-IL-6) for cooperative interaction with NF-κB for IL-8 gene expression in respiratory syncytial virus-infected A549 cells (Mastronarde *et al.*, 1998). However, when this same cell line is stimulated with *Pseudomonas* nitrite reductase (PNR), activation of NF-κB alone is sufficient for maximal IL-8 gene transcription (Mori *et al.*, 1999). Such differences in IL-8 regulation may help to explain some of the inconsistencies observed in studies examining IL-8 production by airway epithelial cell lines in response to particular stimuli. It has been demonstrated on many occasions that stimulation of airway epithelial cells with *Pseudomonas aeruginosa* or certain *Pseudomonas* products, such as PNR or N-3-oxododecanoyl homoserine lactone (3-O-C12-HSL), results in increased expression of IL-8 in cells with CFTR dysfunction compared with matched controls (DiMango *et al.*, 1995; Bryan *et al.*, 1998; Tabary *et al.*, 1999). However, studies looking at the effects of proinflammatory cytokines on airway epithelial IL-8 production have produced conflicting data (Ruef *et al.*, 1993; Stecenko *et al.*, 1997; Black *et al.*, 1998; Schwiebert *et al.*, 1999; Massengale *et al.*, 1999; Venkatakrishnan *et al.*, 2000). Even in this study, the IL-8 profiles generated by cytokine stimulated 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines contrasted with those produced by the cytokine stimulated pCEP cell lines. Kube *et al.* (2000) examined IL-8 production in matched pairs of airway

epithelial cell lines and confirmed that CF phenotype cells generated significantly more IL-8 than control lines in response to *Pseudomonas* stimulation. They also demonstrated prolonged IL-8 production by the CF phenotype cells compared with the control cells. When the same cells were stimulated with a mixture of IL-1β and TNFα the CF phenotype cells again produced more IL-8 than the non-CF cells. However, in this instance, the difference in IL-8 levels between the two cell types was considerably less than the difference observed after *Pseudomonas* stimulation. In addition, no disparity in the time course of IL-8 production was reported between cytokine stimulated CF and non-CF cell lines. The results of this study lend credence to the hypothesis that different signal transduction pathways and ultimately different transcription factors, are activated in response to the stimulation of airway epithelial cells with bacteria compared with stimulation by proinflammatory cytokines. It may even be the case that similar signal transduction pathways are activated in response to IL-1β and TNFα stimulation in normal and CF phenotype cells, whereas the mix of pathways may be different in normal and CF lines in response to *Pseudomonas* stimulation. It has also been suggested that CFTR function has a significant effect on signal transduction pathways activated by *Pseudomonas* stimulation, whilst having little effect on more general IL-8 induction pathways, such as those activated by proinflammatory cytokines (Black *et al.*, 1998). The difference in cytokine stimulated IL-8 levels between CF and non-CF cells may vary depending upon the cell line used and this may shed some light on why some researchers report increased IL-8 production by CF airway epithelial cells in response to cytokine stimulation, whereas others do not.

The fact that the pCEP airway epithelial cell lines used in this study showed increased IL-8 production by the CF phenotype cells compared with the normal phenotype cells,

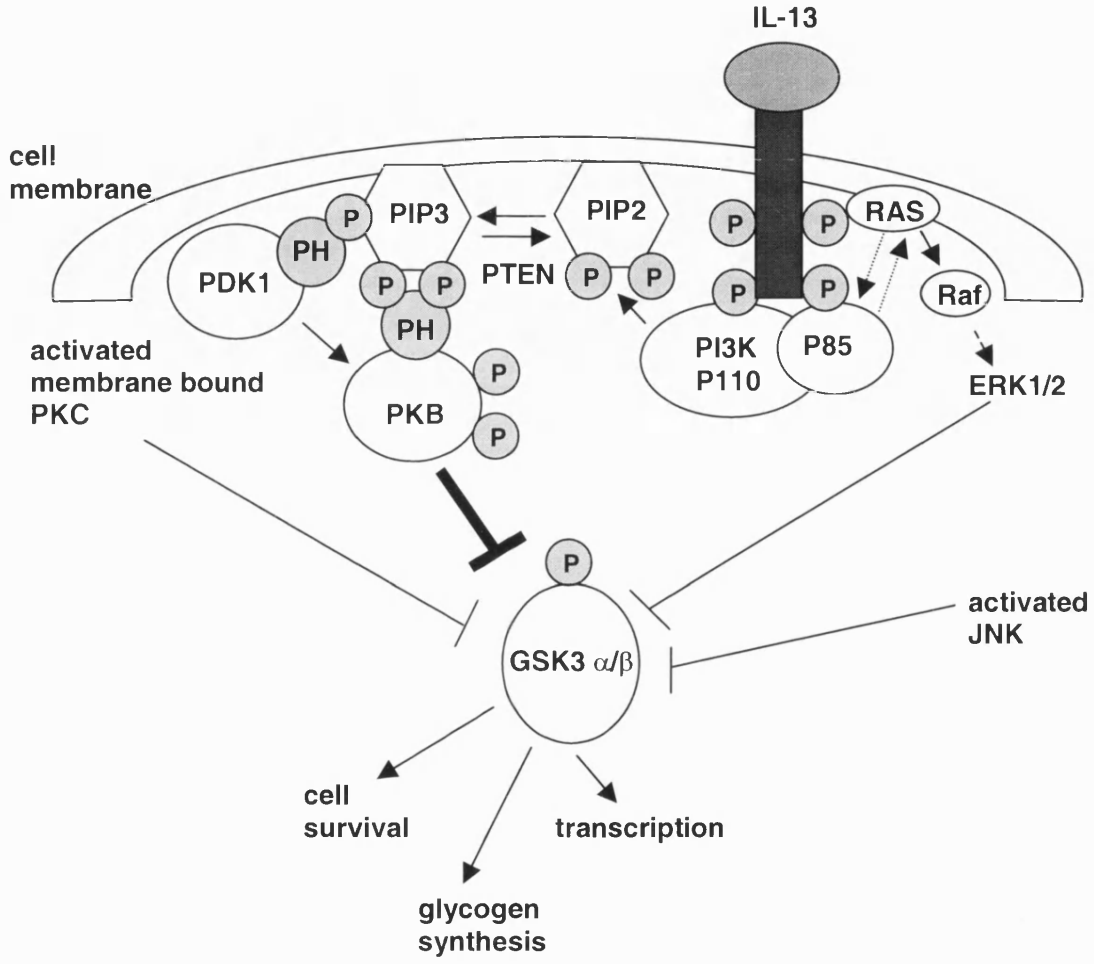
suggests that the presence of functional CFTR may influence cytokine stimulated IL-8 production at some level in these particular cell lines. The CF phenotype 9HTEo$^-$ /pCEPRF cells also produced significantly more IL-8 in the absence of cytokine stimulation than the non-CF 9HTEo$^-$/pCEP#2 cells. This finding seems to lend support to the theory that NF-κB is constitutively active in CF airway epithelial cells compared with normal cells (DiMango *et al.*, 1998; Tabary *et al.*, 1999). However, the use of the pCEP cell lines argues against the ER overload hypothesis. This hypothesis states that aberrant trafficking of mutant CFTR and its accumulation in the endoplasmic reticulum (ER) overwhelms normal cell degradation pathways, provoking stress activation of NF-κB (DiMango *et al.*, 1998). Mutant CFTR is not present in either of the two pCEP cell lines. In these cells CFTR is processed normally and is present at the apical membrane (Perez *et al.*, 1996). Thus, the data generated in this study suggests that the excess cytokine responses are a consequence of impaired cAMP-dependent chloride transport. Venkatakrishnan *et al.* (2000) also refute the ER overload hypothesis and demonstrated that alterations in NF-κB activity in CF airway epithelial cells were associated with changes in IκBβ regulation. After cell stimulation members of the IκB family are phosphorylated, polyubiquitinated and degraded by the 26S proteosome, enabling NF-κB to translocate to the nucleus (reviewed by Barnes & Karin, 1997). Activated nuclear NF-κB causes an upregulation of IκBα mRNA levels by binding to κB motifs in the IκBα promoter (Arenzana-Seisdedos *et al.*, 1995). The newly synthesised IκBα helps terminate the NF-κB response by resequestering NF-κB in the cytoplasm. In quiescent cells IκBβ exists as a basal phosphorylated form that, like IκBα, masks the nuclear localisation signals on NF-κB. After proteosome degradation it is resynthesised as an unphosphorylated (hypophosphorylated) form (Suyang *et al.*, 1996). Unlike IκBα and the basally phosphorylated form of IκBβ,

hypophosphorylated IκBβ is unable to mask the nuclear localisation signal and the DNA binding domain of NF-κB (Suyang *et al.*, 1996). Therefore, NF-κB bound to hypophosphorylated IκBβ is protected from inactivation by IκBα and can enter or remain in the nucleus and mediate persistent transcriptional activation of proinflammatory genes. Venkatakrishnan and colleagues (2000) showed that stimulation of airway epithelial cells with TNFα resulted in IκBβ degradation and accumulation of hypophosphorylated IκBβ, which was associated with prolonged NF-κB activation. However, CF epithelial cells had augmented production of hypophosphorylated IκBβ and increased nuclear NF-κB after TNFα treatment compared with normal and corrected epithelial cell lines. These increased levels of hypophosphorylated IκBβ in the CF epithelial cells correlated with increased IL-8 production. It is possible that the increased production of IL-8 by 9HTEo$^-$/pCEPRF cells after cytokine stimulation was associated with changes in IκBβ regulation and these changes may somehow have been related to impaired cAMP-dependent chloride ion transport. However, it should be noted that this theory does not account for the higher IL-8 levels observed in the absence of cytokine stimulation, when IκBβ would have been present in its phosphorylated form.

Large numbers of macrophages and neutrophils are present in the inflamed CF lung. However, despite this invading bacteria are not eradicated. High concentrations of elastase and reactive oxygen species, such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), are released as a result of the inefficient phagocytosis and lysis of microbes. This creates a highly oxidative environment in the CF airway (Tummler & Kiewitz, 1999). In many systems oxidative stress has been shown to increase cytokine-mediated iNOS expression (Kuo *et al.*, 1997). It has also been demonstrated that

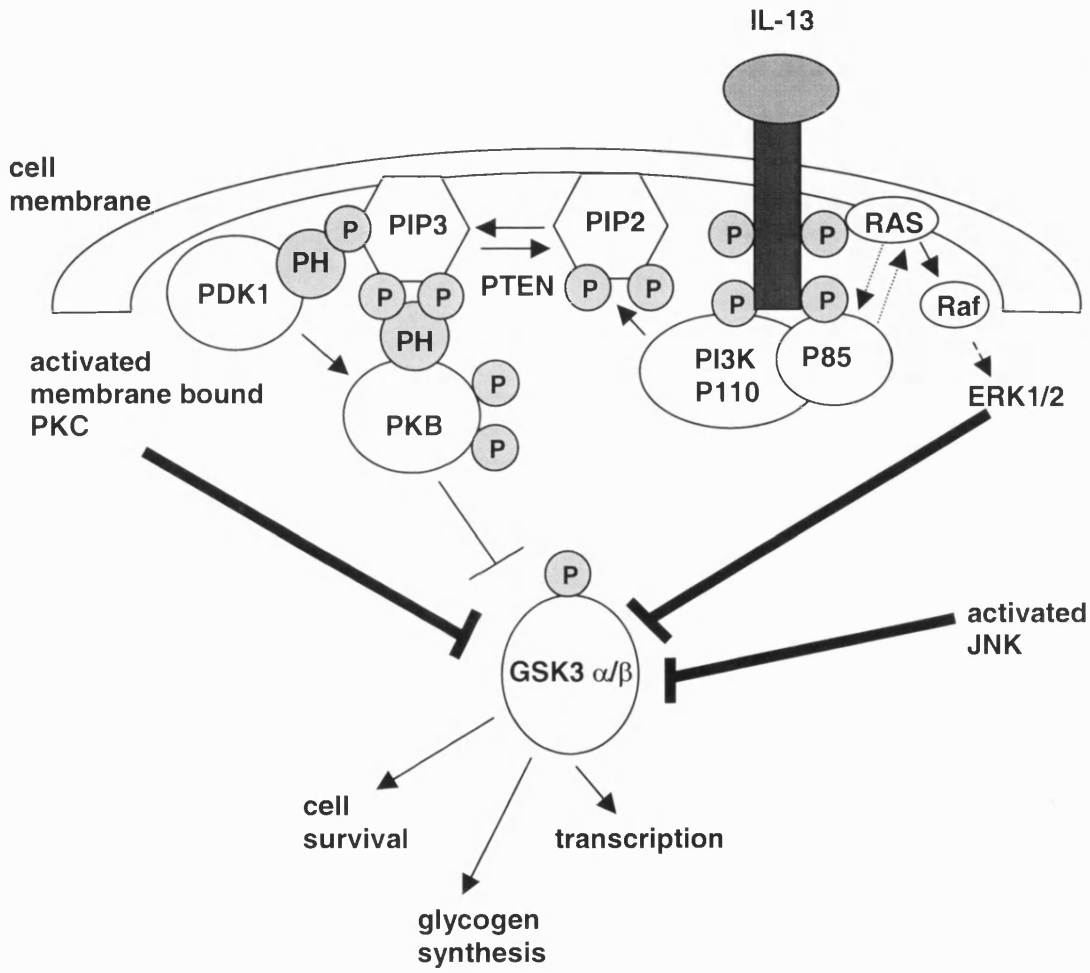
reactive oxygen intermediates induce the activation of NF-κB (Hoare *et al.*, 1999). However, some oxidants, including H_2O_2, have been found to activate the PI3K pathway (Shaw *et al.*, 1998; Sonoda *et al.*, 1999) and inhibition of PI3K has been shown to be necessary for the expression of iNOS in a variety of systems (Donaldson *et al.*, 1996; Park *et al.*, 1997; Wright *et al.*, 1997). It is therefore possible that the highly oxidative environment of the CF lung may lead to activation of PI3K in the airway epithelium, which in turn down regulates iNOS expression. Unfortunately this study was unable to establish any such link between iNOS expression and PI3K activation in the 9HTEo$^-$, ΣCFTE29o$^-$, 9HTEo$^-$/pCEP#2 and 9HTEo$^-$/pCEPRF cell lines due to the lack of nitrite production by these cells in response to proinflammatory cytokine stimulation. Despite this, some interesting differences in the regulation of PI3K/PKB/GSK3 signalling were observed between the 9HTEo$^-$ and ΣCFTE29o$^-$ cell lines. Phosphorylation of GSK3α/β in 9HTEo$^-$ cells was found to be largely dependent on the PI3K pathway, with a small contribution in regulation from the ERK1/2, JNK and PKC signalling pathways. However, in contrast GSK3α/β phosphorylation in ΣCFTE29o$^-$ cells appeared to show a greater dependence on the ERK1/2, JNK and PKC pathways. These findings are summarised in figure 62 (a & b) and may have an impact on a range of cellular processes, including the regulation of inflammatory genes such as iNOS and the regulation of cell survival pathways.

Figure 62a A model for GSK3α/β phosphorylation in 9HTEo⁻ cells in response to IL-13 stimulation



Abbreviations: (P) phosphate; PIP3, PtdIns-3,4,5-P_3; PIP2, PtdIns-4,5-P_2; PI3K, phosphatidylinositol 3-kinase; PDK1, phosphoinositide dependent kinase 1; PKB, protein kinase B; PH, pleckstrin-homology domain; GSK3, glycogen synthase kinase-3; PTEN, phosphatase and tensin homologue deleted on chromosome ten.

Figure 62b A model for GSK3α/β phosphorylation in ΣCFTE29o⁻ cells in response to IL-13 stimulation



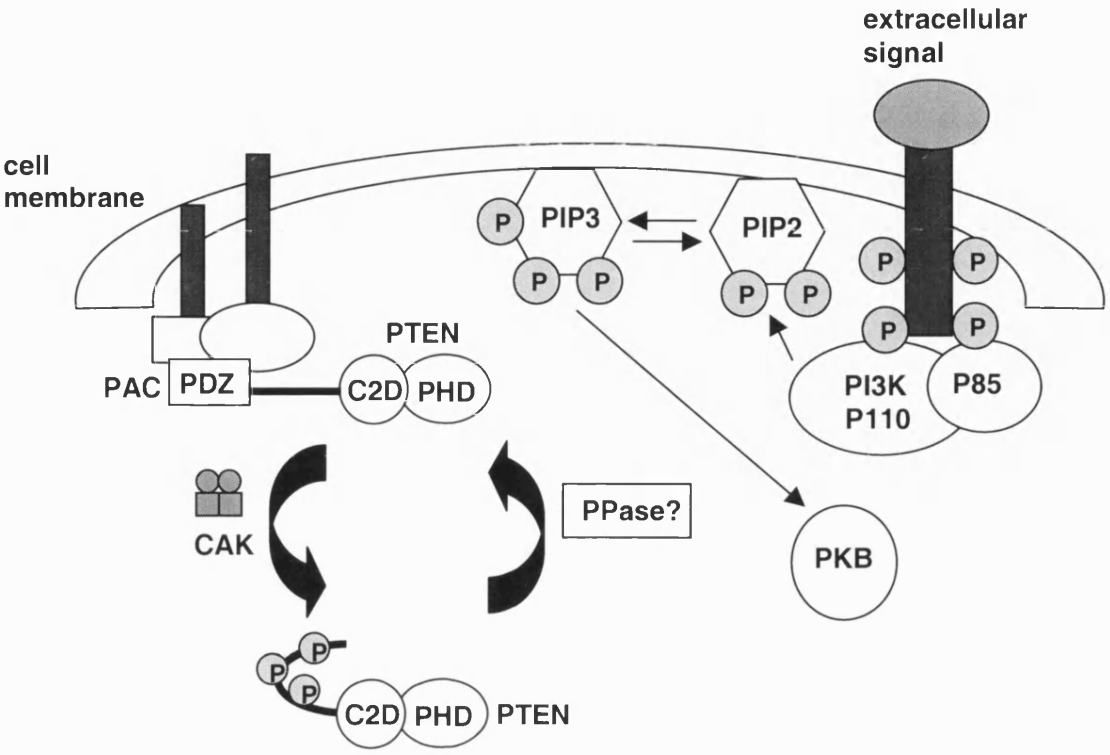
Abbreviations: (P) phosphate; PIP3, PtdIns-3,4,5-P_3; PIP2, PtdIns-4,5-P_2; PI3K, phosphatidylinositol 3-kinase; PDK1, phosphoinositide dependent kinase 1; PKB, protein kinase B; PH, pleckstrin-homology domain; GSK3, glycogen synthase kinase-3; PTEN, phosphatase and tensin homologue deleted on chromosome ten.

It is interesting to note that although the general consensus is that PI3K is required for the activation of NF-κB in most settings, Wright and colleagues (1997) demonstrated that PI3K activation attenuated iNOS expression in HT-29 gut epithelial cells rather than leading to gene induction. No mechanism was proposed to explain the regulation of iNOS expression via PI3K in this study. However, a recent report by Wang and colleagues (2000) has shed some light on these findings. They have demonstrated that inhibition of PI3K in colonic epithelial cells induced NF-κB binding activity and transactivation independently of any effects on IκBα. It may be the case that in some systems PI3K activation affects the phosphorylation status and activity of another protein, which in turn interferes with the iNOS transcriptional machinery. It is also possible that PI3K regulates the transcription of this unknown protein. Such regulatory mechanisms may also operate in other tissues. However, in the absence of a reliable model system for the study of iNOS gene expression in human airway epithelial cells, potential links between PI3K signalling, NF-κB activation and iNOS induction in the context of lung inflammation cannot be fully examined

The role of the lipid phosphatase PTEN in PI3K signalling is very interesting and is worthy of further examination, in that its activity could possibly be linked to the presence of functional CFTR. Although the role of PTEN as a tumour suppressor is well established, the mechanisms by which its lipid phosphatase activity is regulated have only recently begun to be elucidated. PTEN is comprised of an N-terminal phosphatase domain, a C2 domain that binds lipid vesicles and a C-terminal tail that contains a PDZ binding domain (Lee *et al.*, 1999). Several PDZ domain containing proteins have been shown to interact with PTEN, including members of the membrane-associated guanylate kinase (MAGUK) family (Wu *et al.*, 2000). It has been proposed that

MAGUKs function as scaffold proteins to assemble multiprotein signalling complexes and enhance their stability, thereby increasing the efficiency of signal transduction (Pawson & Scott, 1997). In particular, membrane-associated guanylate kinase inverted-2 (MAGI-2) and MAGI-3 have been shown to enhance the activity of PTEN as measured by inhibition of PKB phosphorylation (Wu *et al.*, 2000). Conversely Vazquez & Sellers (2000), have demonstrated that phosphorylation of the PTEN tail by a constitutively active kinase negatively regulates its function as an antagonist of PI3K signalling. More recently Vazquez *et al.* (2001) have shown that unphosphorylated PTEN is found as part of a high molecular weight complex associated with the PDZ domain containing proteins MAGI-2 and MAGI-3. They named this complex the PTEN-associated complex (PAC). Vazquez and colleagues (2001) suggest that recruitment into the PAC is important for localisation of PTEN to the plasma membrane where its substrate lies. They also propose that phosphorylated PTEN exists in a closed conformation and is unable to interact with PDZ domain containing proteins. Phosphorylated PTEN is therefore unable to locate to the plasma membrane. Vazquez *et al.* (2001) hypothesise that as phosphorylation of the PTEN tail is regulated by a constitutively active kinase, the rate-limiting step in switching between phosphorylated and unphosphorylated PTEN is governed by a phosphatase. This phosphatase, upon activation, presumably dephosphorylates the PTEN tail changing its conformation and thus increasing the amount of PTEN available for the PAC (Fig 63). However, in order to confirm this model of PTEN regulation this putative phosphatase as well as its cellular location will need to be identified and its relationship to activated PI3K investigated.

Figure 63 A model for phosphorylation-dependent regulation of PTEN activity (adapted from Vazquez *et al.*, 2001)



Abbreviations: (P) phosphate; PIP3, PtdIns-3,4,5-P_3; PIP2, PtdIns-4,5-P_2; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PTEN, phosphatase and tensin homologue deleted on chromosome ten; C2D, C2 domain; PHD, phosphatase domain; PDZ, PSD-95, DLG, ZO-1 domain; PAC, PTEN associated complex; CAK, constitutively active kinase; PPase?, putative phosphatase

PTEN and CFTR both contain PDZ binding domains (Lee *et al.*, 1999; Moyer *et al.*, 1999; Moyer *et al.*, 2000; Milewski *et al.*, 2001) and are therefore both capable of interacting with PDZ domain containing proteins such as members of the MAGUK family. MAGUK proteins are believed to play a major role in signal transduction through

the clustering of protein complexes at critical structures in the membrane such as the cytoplasmic tails of transmembrane receptors and ion channels (Kornau *et al.*, 1995; Niethammer *et al.*, 1996). It is therefore possible that the PAC into which unphosphorylated PTEN is recruited is linked in some way to membrane-associated CFTR. An absence of functional CFTR in the apical membrane of CF airway epithelial cells could consequently result in a lack of PTEN recruitment to the plasma membrane and a failure to regulate PI3K signalling efficiently. The results of this study confirm that PTEN is present in 9HTEo$^-$ and ΣCFTE29o$^-$ cells and preliminary data suggest that basal PTEN activity is similar between these two cell lines. However, further investigation into the activity of PTEN and its relationship to CFTR in airway epithelial cells may help to provide a broader picture of PI3K signalling and may help to identify mechanisms behind some of the clinical manifestations observed in CF airway inflammation.

7 CONCLUSIONS

In this study, human airway epithelial cells were found to produce high levels of the chemokine IL-8 in response to stimulation with a combination of the proinflammatory cytokines IFNγ, IL-1β and TNFα. These findings provide support for the suggestion that the epithelium is a potentially major source of IL-8 in the airways and implicate CF epithelia at some level in the neutrophil migration observed in CF associated airway inflammation. The Th2 derived cytokine, IL-13 did not affect airway epithelial IL-8 generation and the regulation of IL-8 production was found to be independent of PI3K activity. Cytokine-induced IL-8 production by the CF phenotype cell line 9HTEo$^-$/pCEPRF was found to be significantly increased compared with the genetically matched non-CF control cell line 9HTEo$^-$/pCEP#2. These results lend credence to the theory that CF airway epithelium is proinflammatory compared with normal airway epithelium and suggest that this may be a consequence of impaired cAMP-dependent chloride ion transport.

Human airway epithelial cells were found to produce basal levels of nitrite that were not increased by inflammatory stimuli. These findings suggest that iNOS cannot be induced by proinflammatory cytokines in the particular airway epithelial cell lines used in this study. The lack of response was not a consequence of substrate limitation or substrate competition and may well be a result of immortalisation procedures. It was not possible to demonstrate any links between IL-13, PI3K and NO production in these cells due to the inability to induce nitrite generation above basal levels. It therefore appears that immortalised airway epithelial cell lines may be poor models for the study of human airway epithelial iNOS expression.

IL-13 was shown to activate PI3K and lead to the phosphorylation of PKB and GSK3 in human airway epithelial cells. This has been demonstrated in a range of other systems, but until now had not been confirmed in human airway epithelium. No differences in PI3K activity or levels of PKB and GSK3 phosphorylation were observed between CF and non-CF airway epithelial cell lines. However, some discrepancies in GSK3 regulation were revealed. The ERK1/2, JNK and PKC pathways were shown to play a larger role in GSK3 phosphorylation in a CF airway epithelial cell line than a non-CF airway epithelial cell line. These findings could have implications for the control of the many cellular processes that are regulated by GSK3, such as transcription factor activation and the regulation of cell survival pathways.

In order to investigate fully the possible links between PI3K signalling and the regulation of iNOS, it will be necessary to develop a reliable model system in which the conditions for iNOS expression in human airway epithelial cells are fully characterised. A putative link between PI3K signalling, PTEN and CFTR is also of interest and should be explored further. Once the connections between signal transduction pathways, transcription factor activation and the production of inflammatory mediators such as iNOS and IL-8 are established, it may be possible to manipulate these pathways in order to restore an inflammatory balance in the CF airway. It may also be useful to examine the relationship between GSK3 inactivation and the triggering of cell survival pathways in airway epithelial cells, in the light of suggestions that cell survival mechanisms may be altered in CF.

8 APPENDIX

8.1 SOLUTIONS FOR IMMUNOBLOT ANALYSIS

Lysis Buffer

50 mM TRIS-HCl, pH 7.5

150 mM NaCl

5 mM EDTA, pH 8.0

1% Nonidet P40

10% glycerol

1 mM Na_3VO_4

1 mM $NaMoO_4$

10 mM NaF

40 μg/ml PMSF

0.7 μg/ml pepstatin A

10 μg/ml aprotinin

10 μg/ml leupeptin

10 μg/ml soyabean trypsin inhibitor

5X Sample Buffer

1 M TRIS-HCl, pH 6.8

10% SDS

50% glycerol

5% 2-Mercaptoethanol

0.01 g Bromophenol blue (Bio-Rad) (just enough to give a dark blue colour)

7.5% w/v Acrylamide Running Gel (10ml)

3.75ml Protogel {30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide} (National Diagnostics, Hull, UK)

1 M TRIS-HCl, pH 8.8

0.1% SDS

0.1% ammonium persulphate

5.6 ml Milli-Q water

20 µl N,N,N',N',tetramethylene-diamine (TEMED)

5% w/v Acrylamide Stacking Gel (8ml)

1.33 ml Protogel {30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide} (National Diagnostics, Hull, UK)

1 M TRIS-HCl, pH 6.8

0.1% SDS

0.1% ammonium persulphate

6.36 ml Milli-Q water

20 µl N,N,N',N',tetramethylene-diamine (TEMED)

8.2 SOLUTIONS FOR *IN VITRO* LIPID KINASE ASSAY

Lysis Buffer

20 mM TRIS

137 mM NaCl

1 mM $MgCl_2$

1 mM $CaCl_2$

10% glycerol

1% NP-40

100 µM Na_3VO_4

1 mM PMSF

2 µl/ml aprotinin

2 µl/ml leupeptin

0.4 µl/ml pepstatin A

8.3 SOLUTIONS FOR *IN VITRO* LIPID PHOSPHATASE ASSAY

Lysis Buffer

20 mM TRIS	1% NP-40
137 mM NaCl	1 mM PMSF
1 mM $MgCl_2$	2 µl/ml aprotinin
1 mM $CaCl_2$	2 µl/ml leupeptin
10% glycerol	0.4 µl/ml pepstatin A

9 REFERENCES

Acaro, A., Volina, S., Zvelebil, M.J., Steiner, R., Watton, S.J., Layton, M.J., Gout, I., Ahmad, K., Downward, J., Waterfield, M.D. (1998) Human phosphoinositide 3-kinase C2β and the role of calcium and the C2 domain in enzyme activity. *J.Biol.Chem.*, **273:** 33082-33090.

Adams, D.H., Shaw, S. (1994) Leucocyte-endothelial interactions and regulation of leucocyte migration. *Lancet*, **343:** 831-836.

Adams, L.B., Hibbs, J.B.J., Tainter, R.R., Krahenbuhl, J.L. (1990) Microbiostatic effect of murine-activated macrophages for *Toxoplasma gondii. J.Immunol.*, **144:** 2725-2729.

Akabas, M.H. (2000) Cystic fibrosis transmembrane conductance regulator. Structure and function of an epithelial chloride channel. *J. Biol. Chem.*, **275:** 3729-3732.

Alderton, W.K., Cooper, C.E., Knowles, R.G. (2000) Nitric oxide synthases: structure, function and inhibition. *J.Biochem.*, **357:** 593-615.

Alessi, D.R., Andjelkovich, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., Hemmings, B.A. (1996) Mechanisms of activation of protein kinase B by insulin and IGF-1. *EMBO.J.*, **15:** 6541-6551.

Alpert, D., Schwenger, P., Han, J., Vilcek, J. (1999) Cell stress and MKK6b-mediated p38 MAP kinase activation inhibit tumor necrosis factor-induced IκB phosphorylation and NF-κB activation. *J.Biol.Chem.*, **274:** 22176-22183.

Antwerp, D.J.V., Martin, S.J., Kafri, T., Green, D.R., Verma, I.M. (1996) Suppression of TNF-alpha induced apoptosis by NF-kappaB. *Science*, **274:** 787-789.

Arenzana-Seisdedos, F., Thompson, J., Rodriguez, M.S., Bachelerie, F., Thomas, D., Hay, R.T. (1995) Inducible nuclear expression of newly synthesised IκBα negatively regulated DNA-binding and transcriptional activities of NF-κB. *Mol.Cell.Biol.*, **15:** 2659-2696.

Armstrong, D.S., Grimwood, K., Carlin, J.B., Carzino, R., Gutierrez, J.P., Hull, J., Olinsky, A., Phelan, E.M., Robertson, C.F., Phelan, P.D. (1997) Lower airways inflammation in infants and young children with cystic fibrosis. *Am.J.Respir.Crit.Care Med.*, **156:** 1197-1204.

Armstrong, D.S., Grimwood, K., Carzino, R. (1995) Lower respiratory infection and inflammation in infants with newly diagnosed cystic fibrosis. *Brit.Med.J.*, **310:** 1571-1577.

Asano, K., Chee, C.B.E., Gaston, B., Lilly, C.M., Gerard,C., Drazen, J.M., Stonler, J.S. (1994) Constitutive and inducible nitric oxide synthase gene expression, regulation and activity in human lung epithelial cells. *Proc.Natl.Acad.Sci.USA.*, **91:** 10089-10093.

Assreuy, J., Cunha, F.Q., Epperlein, M., Noronha-Dutra, A., O'Donell, C.A., Liew, F.Y., Moncada, S. (1994) Production of nitric oxide and superoxide by activated macrophages and killing of *Leishmania major. Eur.J.Immunol.*, **24:** 672-676.

Baeuerle, P.A., Baltimore, D. (1996) NF-κB: ten years after. *Cell*, **87:** 13-20.

Baird, R.M., Smith, A.W., Brown, H., Watson, M.L. (1999) Antimicrobial activity and β-defensin-2 expression in human airway epithelial cells. *Immunopharmacol.*, **144:** 267-272.

Baggiolini, M., Dewald, B., Moser, B. (1994) Interleukin-8 and related chemotactic cytokines – CXC and CC chemokines. *Adv.Immunol.*, **55:** 97-179.

Baggiolini, M., Walz, A., Kunkel, S.L. (1989) Neutrophil-activating peptide-1 / interleukin-8, a novel cytokine that activates neutrophils. *J.Clin.Invest.*, **84:** 1045-1049.

Baldwin, A.S. (1996) The NF-κB and IκB proteins: new discoveries and insights. *Annu.Rev.Immunol.*, **14:** 649-681.

Baldwin, A.S. (2001) The transcription factor NF-κB and human disease. *J.Clin.Invest.*, **107:** 3-11.

Balfour-Lynn, I.M., Laverty, A., Dinwiddie, R. (1996) Reduced upper airway nitric oxide in cystic fibrosis. *Arch.Dis.Childhood*, **75:** 319-322.

Balough, K., McCubbin, M., Weinberger, M., Smits, W., Ahrens, R., Fick, R. (1995) The relationship between infection and inflammation in the early stages of lung disease from cystic fibrosis. *Ped.Pulmonol.*, **20:** 63-70.

Bals, R., Weiner, D.J., Wilson, J.M. (1999) The innate immune system in cystic fibrosis lung disease. *J.Clin.Invest.*, **103:** 303-307.

Barnes, P.J., Karin, M. (1997) Nuclear factor-kappaB – a pivotal transcription factor in chronic inflammatory diseases. *N.Engl.J.Med.*, **336:** 1067-1071.

Barnes, P.J., Liew, F.Y. (1995) Nitric oxide and asthmatic inflammation. *Immunol.Today*, **16:** 128-129.

Bauernfeind, A., Bertele, R., Harms, K. (1997) Qualitative and quantitative microbiological analysis of sputa of 102 patients with cystic fibrosis. *Infection*, **15:** 270-278.

Becker, S., Koren, H.S., Henke, D.C. (1993) Interleukin-8 expression in normal and nasal epithelium and its modulation by infection with respiratory syncytial virus and cytokines tumour necrosis factor, interleukin-1 and interleukin-6. *Am.J.Respir.Cell Mol.Biol.*, **8:** 20-27.

Bevins, C.L. (1999) Scratching the surface. Inroads to a better understanding of airway host defense. *Am.J.Respir.Cell Mol.Biol.*, **20:** 861-863.

Bird, T.A., Schooley, K., Dower, S.K., Hagen, H., Virca, G.D. (1997) Activation of nuclear transcription factor NF-kappa B by interleukin-1 is accompanied by casein kinase II-mediated phosphorylation of the p65 subunit. *J.Biol.Chem.*, **272:** 32606-32612.

Birrer, P., McElvaney, N.G., Rudeberg, A., Sommer, C.W., Liechte-Gallati, S., Kraemer, R., Hubbard, R., Crystal, R.G. (1994) protease-antiprotease imbalance in the lungs of children with cystic fibrosis. *Am.J.Respir.Crit.Care Med.*, **150:** 207-213.

Black, H.R., Yankaskas, J.R., Johnson, L.G., Noah, T.L. (1998) Interleukin-8 production by cystic fibrosis nasal epithelial cells after tumor-necrosis factor-α and respiratory syncytial virus stimulation. *Am.J.Respir.Cell Mol.Biol.*, **19:** 210-215.

Blenis, J. Resh, M.D. (1993) Subcellular localisation specified by protein acylation and phosphorylation. *Curr.Opin.Cell Biol.*, **5:** 984-989.

Boat, T.F., Welsh, M.J., Beaudet, A.L. (1989) The metabolic basis of inherited disease. 2649-2680 McGraw-Hill.

Bonfield, T.L., Panuska, J.R., Konstan, M.W. (1995) Inflammatory cytokines in cystic fibrosis lungs. *Am.J.Respir.Crit.Care Med.*, **152:** 2111-2118.

Bories, P.N., Campillo, B., Scherman, E. (1999) Upregulation of nitric oxide production by interferon-gamma in cultured peritoneal macrophages from patients with cirrhosis. *Clin.Sci.*, **97:** 399-406.

Borish, L., Mascali, J.J., Dishuck, J., Beam, W.R., Martin, R.J., Rosenwasser, L.J. (1992) Detection of alveolar macrophage-derived IL-1 beat in asthma. Inhibition with corticosteroids. *J.Immunol.*, **149:** 3078-3082.

Bousquet, J., Chonez, P., Lacoste, J.Y. (1990) Eosinophilic inflammation in asthma. *N.Eng.J.Med.*, **323:** 1033-1039.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal.Biochem.*, **72:** 248-254.

Brian, J.E., Faraci, F.M., Heistad, D.D. (1996) Recent insights into the regulation of cerebral circulation. *Clin.Exp.Pharmacol.Physiol.*, **23:** 449-457.

Brombacher, F. (2000) The role of interleukin-13 in infectious diseases and allergy. *BioEssays*, **22:** 646-656.

Brunet, A., Bonn, A., Zigond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., Greenberg, M.E. (1999) Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. *Cell*, **96:** 857-868.

Bryan, R., Kube, D., Perez, A., Davis, P.B., Prince, A. (1998) Overproduction of the CFTR R-domain leads to increased levels of asialo GM_1 and increased *P. aeruginosa* binding by epithelial cells. *Am.J.Respir.Cell Mol.Biol.*, **19:** 269-277.

Cantin, A. (1995) Cystic fibrosis lung inflammation: early, sustained and severe. *Am.J.Respir.Crit.Care Med.*, **151:** 939-941.

Cantley, L.C., Neel, B.G. (1999) New insights into tumour suppression: PTEN suppresses tumour formation by restraining the phosphoinositide 3-kinase/Akt pathway. *Proc.Natl.Acad.Sci.USA.*, **96:** 4240-4245.

Cardone, M.H., Roy, N., Sternicke, H.R., Salvasen, G.S., Franke, T.F., Stanbridge, E., Frisch, S., Reed, J.C. (1998) Regulation of cell death protease caspase-9 by phosphorylation. *Science*, **282:** 1318-1321.

Cattell, V., Cook, H.T. (1993) Nitric oxide. Role in the physiology and pathology of the glomerulus. *Exp.Nephrol.*, **5:** 265-280.

Chang, C.I., Liao, J.C., Kuo, L. (1998) Arginase modulates nitric oxide production in activated macrophages. *Am.J.Physiol.*, **274:** H342-H348.

Charles, I.G., Palmer, R.M., Hickery, M.S., Bayliss, M.T., Chubb, A.P., Hall, V.S., Moss, D.W., Moncada, S. (1993) Cloning, characterisation and expression of a cDNA encoding an inducible nitric oxide synthase from the human chondrocyte. *Proc.Natl.Acad.Sci.USA.*, **90:** 11419-11423.

Chartrain, N.A., Geller, D.A., Koty, P.P., Sitrin, N.F., Nussler, A.K., Hoffman, E.P., Billiar, T.R., Hutchinson, N.I., Mudgett, J.S. (1994) Molecular cloning, structure and chromosomal localisation of the human inducible nitric oxide synthase gene. *J.Biol.Chem.*, **269:** 6765-6772.

Chen, Z.J., Parent, L., Maniatis, T. (1996) Site-specific phosphorylation of IκBα by a novel ubiquitin-dependent protein kinase activity. *Cell*, **84:** 853-862.

Cheng, S.H., Gregory, R.J., Marshall, J., Paul, S., Souza, D.W., White, G.A., O'Riordan, C.R., Smith, A.E. (1990). Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell*, **63:** 827-834.

Cheng, S.H., Rich, D.P., Arshall, J., Gregory, R.J., Welsh, M.J., Smith, A.E. (1991) Phosphorylation of the R domain by cAMP-dependent protein kinase regulates the CFTR chloride channel. *Cell*, **66:** 1027-1036.

Chollet-Martin, S., Montravers, P., Gilbert, C. (1993) High levels of interleukin-8 in the blood and alveolar spaces of patients with pneumonia and adult respiratory distress syndrome. *Infect.Immun.*, **61:** 4553-4559.

Chu, S.C., Marks, K.J., Wu, H.P., Banks, T.C., Moss, J. (1998) Analysis of the cytokine-stimulated human inducible nitric oxide synthase (iNOS) gene: characterisation of differences between human and mouse iNOS promoters. *Biochem.Biophys.Res.Commun.*, **248:** 871-878.

Coffer, P.J., Jin, J., Woodgett, J.R. (1998) Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem.J.*, **335:** 1-13.

Cole, A.M., Dewan, P., Ganz,T. (1999) Innate antimicrobial activity of nasal secretions. *Infect.Immun.*, **67:** 3267-3275.

Collins, F.S. (1992) Cystic fibrosis: molecular biology and therapeutic implications. *Science*, **256:** 774-779.

Colten, H.R. (1995) Airway inflammation in cystic fibrosis. *New.Engl.J.Med.*, **332:** 886-887.

Consentino, F., Luscher, T.F. (1995) Maintenance of vascular integrity: role of nitric oxide and other bradykinin mediators. *Eur.Heart J.*, **16:** 4-12.

Corvera, S., Czech, M.P. (1998) Direct targets of phosphoinositide 3-kinase products in membrane trafficking and signal transduction. *Trends Cell Biol*, **8:** 442-446.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korbe, D.R., Lappin-Scott, H.M. (1995) Microbial biofilms. *Annu.Rev.Microbiol.*, **49:** 711-745.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., Hemmings, B.A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated protein kinase B. *Nature*, **378:** 785-789.

Cunha, F.Q., Moncada, S., Liew, F.Y. (1992) Interleukin-10 (IL-10) inhibits the induction of nitric oxide synthase by interferon-gamma in murine macrophages. *Biochem.Biophys.Res.Commun.*, **182:** 1155-1159.

Dakin, C.J., Numa, A.H., Wang, H.E., Martan, J.R., Vertzyas, C.C., Henry, R.L. (2002) Inflammation, infection and pulmonary function in infants and young children with cystic fibrosis. *Am.J.Respir.Crit.Care Med.*, **165:** 904-910.

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., Greenberg, M.E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell*, **91**: 231-241.

DeGroote, M.A., Granger, D., Xu, Y., Campbell, G., Prince, R., Fang, F.C. (1995) Genetic and redox determinants of nitric oxide cytotoxicity in a *Salmonella typhimurium* model. *Proc.Natl.Acad.Aci.USA.*, **92:** 6399-6403.

Del Pesso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., Nunez, G. (1997) Interleukin-3 induced phosphorylation of BAD through the protein kinase Akt. *Science*, **278:** 687-689.

DeVera, M.E., Shapiro, R.A., Nussler, A.K., Mudgett, J.S., Simmons, R.L., Morris, S.M., Billiar, T.R., Geller, D.A. (1996) Transcriptional regulation of human inducible nitric oxide synthase (NOS2) gene by cytokines: initial analysis of the human NOS2 promoter. *Proc.Natl.Acad.Sci.USA.*, **93:** 1054-1059.

Diamond, G., Bevins, C.L. (1998) β-defensins: endogenous antibiotics of the innate host defence response. *Clinic.Immunol.Immunopathol.*, **88:** 221-225.

Diamond, G., Legarda, D., Ryan, L. (2000) The innate immune response of the respiratory epithelium. *Immunol.Rev.*, **173:** 27-38.

Diamond, G., Russell, J.P., Bevins, C.L. (1996) Inducible expression of an antibiotic peptide gene in lipopolysaccharide-challenged tracheal epithelial cells. *Proc.Natl.Acad.Sci.USA.*, **93:** 5156-5160.

DiCristofano, A., Kotsi, P., Peng, Y.F., Cordon-Cordo, C., Elkon, K.B., Pandolfi, P.P. (1999) Impaired Fas response and autoimmunity in PTEN +/- mice. *Science*, **285:** 2122-2125.

DiDonato, J., Mercurio, F., Rosette, C. (1996) Mapping of the inducible IκB phosphorylation sites that signal its ubiquitination and degradation. *Mol.Cell Biol.*, **16:** 1295-1304.

DiMango, E., Ratner, A.J., Bryan, R., Tabibi, S., Prince, A. (1998) Activation of NF-κB by adherent *Pseudomonas aeruginosa* in normal and cystic fibrosis respiratory epithelial cells. *J.Clin.Invest.*, **101:** 2598-2605.

DiMango, E., Zar, H.J., Bryan, R., Prince, A. (1995) Diverse *Pseudomonas aeruginosa* gene products stimulate respiratory epithelial cells to produce interleukin-8. *J.Clin.Invest.*, **96:** 2204-2210.

Ding, A., Nathan, CF., Graycar, J., Derynk, R. Stuehr, D.J., Srimal, S. (1990) Macrophage deactivating factor and transforming growth factor beta-1, factor beta-2 and factor beta-3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-gamma. *J.Immunol.*, **145:** 940-944.

Doherty, T.M., Kastelein, R., Menon, S., Andrade, S., Coffman, R.L. (1993) Modulation of murine macrophage function by IL-13. *J.Immunol.*, **151:** 7151-7160.

Domin, J., Pages, F., Volina, S., Rittenhouse, S.E., Zvelebil, M.J., Stein, R.C., Waterfield, M.D. (1997) Cloning of a human phosphoinositide 3-kinase with a C2 domain that displays reduced sensitivity to the inhibitor wortmannin. *Biochem.J.*, **326:** 139-147.

Donaldson, A., Daphnalken, D., Tetsuha, T., Morrison, A.R. (1996) Interleukin-1 beta activates PI3-kinase in renal messengial cells. *Biochem.Biophys.Res.Commun.*, **227:** 289-293.

Donnelly, L.E., Barnes, P.J. (2002) Expression and regulation of inducible nitric oxide synthase from human primary airway epithelial cells. *Am.J.Respir.Cell Mol.Biol.*, **26:** 144-151.

Downward, J. (1998) Lipid-regulated kinases: some common themes at last. *Science*, **279:** 673-674.

Doyle, A.G., Herbein, G., Montaner, L.J., Munty, A.J., Caput, D., Ferrara, P., Gordon, S. (1994) Interleukin-13 alters the activation state of murine macrophages *in vitro*: comparison with interleukin-4 and interferon-gamma. *Eur.J.Immunol.*, **24:** 1441-1445.

Eigen, H., Rosentein, B.J., Fitzsimmons, S., Schidlow, D.V. (1995) A multicenter study of alternate-day prednisone therapy in patients with cystic fibrosis. Cystic Fibrosis Foundation Prednisone Trial Group. *J.Pediatr.*, **126:** 515-523.

Elmer, H.L., Brady, K.G., Drumm, M.L., Kelley, T.J. (1999) Nitric oxide mediated regulation of transepithelial sodium and chloride transport in murine nasal epithelium. *Am.J.Physiol.*, **276:** L466-L473.

Embi, N., Rylatt, D.B., Cohen, P. (1980) Glycogen synthase kinase-3 from rabbit skeletal muscle. Seperation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *Eur.J.Biochem.*, **107:** 519-527.

Fang, F.C. (1997) Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. *J.Clin.Invest.*, **99:** 2818-2825.

Felley-Bosco, E., Ambs, S., Lowenstein, C.J., Keefer, L.K., Harris, C.C. (1994) Constitutive expression of inducible nitric oxide synthase in human bronchial epithelial cells induces c-fos and stimulates the cGMP pathway. *Am.J.Respir.Cell Mol.Biol.*, **11:** 159-164.

Fernandes, P.D., Assreuy, J. (1997) Role of nitric oxide and superoxide in *Giardia lamblia* killing. *Braz.J.Med.Biol.Res.*, **30:** 93-99.

Fiol, C.J., Williams, J.S., Chou, C., Wang, Q.M., Roach, P.J., Andrisani, O.M. (1994) A secondary phosphorylation of CREB341 at Ser 129 is required for the cAMP-mediated control of glycogen synthase kinase-3 in the control of gene expression. *J.Biol.Chem.*, **269:** 32187-32193.

Frame, F., Cohen, P. (2001) GSK3 takes centre stage more than 20 years after its discovery. *Biochem.J.*, **359:** 1-16.

Francoeur, C., Dennis, M. (1995) Nitric oxide and interleukin-8 as inflammatory components of cystic fibrosis. *Inflammation*, **19:** 587-598.

Franke, T.F., Kaplan, D.R., Cantley, L.C. (1997) Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Cell*, **88:** 435-437.

Frizzell, R.A., Rechkemmer, G., Shoemaker, R.L. (1986) Altered regulation of airway epithelial cell chloride channels in cystic fibrosis. *Science*, **233:** 558-560.

Fry, M.J. (1994) Structure, regulation and function of phosphoinositide 3-kinases. *Biochim.Biophys.Acta*, **1226:** 237-268.

Ganster, R.W., Taylor, B.S., Shao, L., Geller, D.A. (2001) Complex regulation of human inducible nitric oxide synthase gene transcription by STAT-1 and NF-κB. *Proc.Natl.Acad.Sci.USA.*, **98:** 8638-8643.

Garcia-Zepeda, E.A., Rothenberg, M.E., Ownberg, R.T., Celestin, J., Leder, P., Luster, A.D. (1996) Human eotaxin is a specific chemoattractant for eosinophils cells and provides a new mechanism to explain tissue eosinophilia. *Nat.Med.*, **4:** 449-456.

Geller, D.A., Lowenstein, C.J., Shapiro, R.A., Nussler, A.K., DiSilvo, M., Wang, S.C., Nakayama, D.K., Simmons, R.L., Snyder, S.H., Billiar, T.R. (1993) Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. *Proc.Natl.Acad.Sci.USA.*, **90:** 3491-3495.

Ghoda, L., Lin, X., Greene, W.C. (1997) The 90 kDa ribosomal S6 kinase (pp90rsk) phosphorylates the N-terminal regulatory domain of IκBα and stimulates its degradation *in vitro*. *J.Biol.Chem.*, **272:** 21281-21288.

Gilljam, H., Ellin, A., Strandvik, B. (1989) Increased bronchial chloride concentration in cystic fibrosis. *Scan.J.Clin.Lab.Invest.*, **49:** 121-124.

Gold, M.R., Chan, V.W., Turck, C.W., DeFranco, A.L. (1992) Membrane Ig cross-linking regulates phosphatidylinositol 3-kinase in B lymphocytes. *J.Immunol.*, **148:** 2012-2022.

Gosset, P., Tsicopoulos, A., Wallaert, B., Bannimenus, C., Joseph, M., Tonnel, A.B., Capron, A. (1991) Increased secretion of tumor necrosis factor alpha and interleukin-6 by alveolar macrophages consecutive to the development of the late asthmatic reaction. *J.Allergy Clin.Immunol.*, **88:** 561-571.

Granger, D.L., Hibbs, J.B., Perfect, J.R., Durack, D.T. (1990) Metabolic fate of L-arginine in relation to microbiostatic capability of murine macrophages. *J.Clin.Invest.*, **85:** 264-273.

Grasemann, H., Michler, E., Wallot, M., Ratjen, F. (1997) Decreased concentration of exhaled NO in patients with cystic fibrosis. *Ped.Pulmonol.*, **24:** 173-177.

Green, S.J., Nacy, C.A. (1993) Antimicrobial and immunopathologic effects of cytokine-induced nitric oxide synthesis. *Curr.Opp.Infect.Dis.*, **6:** 384-396.

Grim, M.C., Doe, W.F. (1996) Chemokines in inflammatory bowel disease mucosa: expression of RANTES, macrophage inflammatory protein (MIP)-1α, MIP-1β and γ interferon-inducible protein-10 by macrophages, lymphocytes, endothelial cells and granulomas. *Inflamm.Bowel Dis.*, **2:** 88-96.

Grimes, C.A., Jope, R.S. (2001) The multifaceted roles of glycogen synthase kinase-3 beta in cellular signalling. *Progress in Neurobiol.*, **65:** 391-426.

Grubb, B.R., Taran, R., Randell, S.H., Gatzy, J.T., Davis, C.W., Boucher, R.C. (1998) Evidence for peciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airway disease. *Cell*, **95:** 1005-1015.

Gruenert, D.C., Basbaum, C.B., Welsh, M.J., Li, M., Finkbeiner, W.E., Nadel, J.A. (1998) Characterization of human tracheal epithelial cells transformed by an origin-defective simian virus 40. *Proc.Natl.Acad.Sci.USA.*, **85:** 5951-5955.

Guo, F.H., Comhair, S.A., Zheng, S., Sweik, R.A., Eissa, N.T., Thomassen, M.J., Calhoun, W., Erzurum, S.C. (2000) Molecular mechanism of increased nitric oxide (NO) in asthma: evidence for transcriptional and post-translational regulation of NO synthesis. *J.Immunol.*, **164:** 5970-5980.

Guo, F.H., DeRaeve, H.R., Rice, T.W., Stuehr, D.J., Thunnison, F.B.J.M., Erzurum, S.C. (1995) Continuous nitric oxide synthesis by inducible nitric oxide synthase in normal human airway epithelium *in vivo*. *Proc.Natl.Acad.Sci.USA.*, **92:** 7809-7813.

Guo, F.H., Uetani, K., Haque, S.J., Williams, B.R., Sweik, R.A., Thunnissen F.B., Calhoun, W., Ezurum, S.C. (1997) Interferon gamma and interleukin-4 stimulate prolonged expression of inducible nitric oxide synthase in human airway epithelium through synthesis of soluble mediators. *J.Clin.Invest.*, **100:** 829-838.

Harbitz, O., Jenssen, A.O., Smidsrod, O. (1984) Lysozyme and lactoferrin in sputum from patients with chronic obstructive lung disease. *Eur.J.Respir.Dis.*, **65:** 512-520.

Harris, B.Z., Lim, W.A. (2001) Mechanism and role of PDZ domains in signalling complex assembly. *J.Cell Science*, **114:** 3219-3231.

Herbert, J.M., Savi, P., Laplace, M.C., Lale, A., Dol, F., Dumas, A., Labit, C., Minty, A. (1993) IL-4 and IL-13 exhibit comparable abilities to reduce pyrogen-induced expression of procoagulant activity in endothelial cells and monocytes. *FEBS.Letts.*, **328:** 268-270.

Hoare, G.S., Marczin, N., Chester, A.H., Yacoub, M.H. (1999) Role of oxidant stress in cytokine-induced activation of NF-κB in human aortic smooth muscle cells. *Am.J.Physiol.*, **277:** H1975-H1984.

Hrabak, A., Idei, M., Temesi, A. (1994) Arginine supply for nitric oxide synthesis and arginase is mainly exogenous in elicited murine and rat macrophages. *Life Sci.*, **55:** 797-805.

Hsu, H.L., Schu, H.B., Pan, M.G., Goeddel, D.V. (1996) TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell*, **84:** 299-308.

Hunter, T., Karin, M. (1992) The regulation of transcription by phosphorylation. *Cell*, **70:** 375-387.

Imundo, L., Barasch, J., Prince, A., Al-Awqati, Q. (1995) Cystic fibrosis epithelial cells have a receptor for pathogenic bacteria on their apical surface. *Proc.Natl.Acad.Sci.USA.*, **92:** 3019-3023.

Jacobs, B.L., Langland, J.O. (1996) When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. *Virology*, **219:** 339-349.

Jaff, A., Bush, A. (2001) Cystic fibrosis a review of the decade. *Arch.Chest.Dis.*, **56:** 240-247.

Jeon, Y.J., Han, S.H., Lee, Y.W., Yea, S.S., Yang, K.H. (1998) Inhibition of NF-kappa B/Rel nuclear translocation by dexamethasone: mechanism for the inhibition of iNOS gene expression. *Biochem.Mol.Biol.Int.*, **45:** 435-441.

Jorens, P.G., Vermeire, P.A., Herman, A.C. (1993) L-arginine dependent nitric oxide synthase. A new metabolic pathway in the lung and airways. *Eur.Respir.J.*, **6:** 258-266.

Juedes, M.J., Wogan, G.N. (1996) Peroxynitrite-induced mutation spectra of pSP189 following replication in bacteria and in human cells. *Mutat.Res.*, **349:** 51-61.

Kalin, N., Claa, B., Sommer, M., Puchell, E., Tummler, B. (1999) ΔF508 CFTR protein expression in tissues from patients with cystic fibrosis. *J.Clin.Invest.*, **103:** 1379-1389.

Kaliner, M.A. (1991) Human nasal respiratory secretions and host defence. *Am.Rev.Respir.Dis.*, **144:** 552-556.

Kamosinska, B., Radomski, M.W., Duszyk, M., Radomski, A., Man, S.F.P. (1997) Nitric oxide activates chloride currents in human lung epithelial cells. *Am.J.Physiol.*, **272:** L1098-L1104.

Kane, L.P., Shapiro, V.S., Stokoe, D., Weiss, A. (1999) Induction of NF-kappa B by the Akt/PKB kinase. *Curr.Biol.*, **9:** 601-604.

Kaplan, S.S., Lancaster, J.R., Baford, R.E., Simmons, R.L. (1996) Effect of nitric oxide on *Staphylococcal* killing and interactive effect with superoxide. *Infect.Immun.*, **64:** 69-76.

Kelley, T.J., Drumm, M.L. (1998) Inducible nitric oxide synthase expression is reduced in cystic fibrosis murine and human epithelial cells. *J.Clin.Invest.*, **102:** 1200-1207.

Kerem, B.S., Rommens, J.M., Buchanan, J.A., Markiewicz, D., Cox, T.K., Chadrevarti, A., Buchwald, M., Tsui, L.C. (1989) Identification of the cystic fibrosis gene: genetic analysis. *Science*, **245:** 1073-1080.

Khan, T.Z., Wagner, J.S., Bost, T., Martinez, J., Accurso, F.J., Riches, D.W. (1995) Early pulmonary inflammation in infants with cystic fibrosis. *Am.J.Respir.Crit.Care Med.*, **151:** 1075-1082.

Kharitonov, S.A., Wells, A.U., O'Connor, B.J., Cole, P.J., Barnes, P.J. (1994) Elevated levels of exhaled nitric oxide in bronchiectasis. *Am.J.Respir.Crit.Care Med.*, **149:** 198-203.

Knowles, M.R., Stutts, M.J., Boucher, R.C. (1993) Abnormal ion permeation through cystic fibrosis respiratory epithelium. *Science*, **221:** 1067-1070.

Knowles, R.G., Moncada, S. (1994) Nitric oxide synthases in mammals. *Biochem.J.*, **298:** 249-258.

Knuzelmann, K., Schweibert, E.M., Zeitlin, P.L., Kuo, W.L., Staton, B.A., Gruenert, D.C. (1993) An immortalized cystic fibrosis tracheal epithelial cell line homozygous for the ΔF508 CFTR mutation. *Am.J.Respir.Cell Mol.Biol.*, **8:** 522-529.

Kolios, G., Robertson, D.A.F., Jordan, N.J., Minty, A., Caput, D., Ferrara, P., Westwick, J. (1996) Interleukin-8 production by the human colon epithelial cell line HT-29: modulation by interleukin-13. *Br.J.Pharmacol.*, **119:** 351-359.

Konstan, M.W., Byard, P.J., Hoppel, C.L., Davis, P.B. (1995) Effect of high dose ibuprofen in patients with cystic fibrosis. *N.Eng.J.Med.*, **332:** 848-854.

Korneau, H.C., Schenker, L.T., Kennedy, M.B., Seeburg, P.H. (1995) Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science*, **269:** 1737-1740.

Kristof, A.S., Marks-Konczalik, J., Moss, J. (2001) Mitogen-activated protein kinases mediate activator protein-1-dependent human inducible nitric oxide synthase promoter activation. *J.Biol.Chem.*, **276:** 8445-8452.

Kroncke, K.D., Fehsel, K., Kolb-Bachofen, V. (1998) Inducible nitric oxide synthase in human diseases. *Clin.Exp.Immunol.*, **113:** 147-156.

Krugmann, S., Hawkins, P.T., Pryer, N., Braselmann, S. (1999) Characterising the interactions between the two subunits of the p101/p110 gamma phosphoinositide 3-kinase and their role in the activation of this enzyme by G beta gamma subunits. *J.Biol.Chem.*, **274:** 17152-17158.

Krystal, G. (2000) Lipid phosphatases in the immune system. *Sem.Immunol.*, **12:** 397-403.

Kube, D., Sontich, U., Fletcher, D., Davis, P.B. (2001) Proinflammatory cytokine responses to *P. aeruginosa* infection in human airway epithelial cell lines. *Am.J.Physiol.Lung Cell Mol.Physiol.*, **280:** L493-L502.

Kunsch, C., Rosen, C.A. (1993) NF-κB subunit specific regulation of the interleukin-8 promoter. *Mol.Cell Biol.*, **13:** 6137-6146.

Kuo, P.C., Alfrey, E.J., Drieger, N.R., Abe, K.Y., Huie, P., Sibley, R.K., Dafoe, D.C. (1997) Differential localization of allograft nitric oxide synthesis: comparison of liver and heart transplantation in the rat model. *Immunology*, **87:** 647-653.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227:** 680-685.

Laskin, J.D., Heck, D.E., Laskin, D.L. (1994) Multifunctional role of nitric oxide in inflammation. *TIEM.*, **5:** 377-382.

Lee, J.O., Yang, H., Georgescu, M.M., DiCristofano, A., Maehama, T., Shi, Y., Dixon, J.E., Pandolfi, P., Pavetich, N.P. (1999) Crystal structure of the PTEN tumour suppressor: implications for its phosphoinositide phosphatase activity and membrane association. *Cell*, **99:** 323-334.

Leonard, E.J., Yoshimura, T. (1990) Human monocyte chemoattractant protein-1 (MCP-1). *Immunol.Today*, **11:** 97-101.

Li, D.M., Sun, H. (1997) TEP1 encoded by a candidate tumour suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. *Cancer Res.*, **57:** 2124-2129.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S.H. (1997) PTEN, a putative tyrosine phosphatase gene mutated in human brain, breast and prostate caner. *Science*, **275:** 1943-1947.

Li, M., McCann, J.D., Liedthe, C.M., Nairn, A.C., Greengard, P., Welsh, M.J. (1988) Cyclic AMP-dependent protein kinase opens chloride channels in normal but not cystic fibrosis airway epithelium. *Nature*, **331:** 358-360.

Liew, F.Y. (1995) Interactions between cytokines and nitric oxide. *Adv.Neuroimmun.*, **5:** 201-209.

Light, D.B., Schwiebert, E.M., Karlson, K.H., Stanton, B.A. (1989) Atrial natriuretic peptide inhibits a cation channel in renal inner medullary collecting ducts. *Science*, **243:** 383-385.

Lindley, I.J.D., Westwick, J., Kunkel, S.L. (1993) The chemokines: biology of the inflammatory peptide supergene family (II). *Adv.in Experi.Med.&Biol.*, **351:** 223-228.

Linn, S.C., Morelli, P.J., Dery, I., Cattangim, S.E., Szabo, C., Salzman, A.L. (1997) Transcriptional regulation of human inducible nitric oxide synthase gene in an intestinal epithelial cell line. *Am.J.Physiol.*, **272:** G1499-G1508.

Linnane, S.J., Keating, V.M., Costello, C.M. (1998) Total sputum nitrite is raised during acute pulmonary infection in cystic fibrosis. *Am.J.Respir.Crit.Care Med.*, **158:** 201-212.

Look, D.C., Roswit, W.T., Frick, A.G., Gris-Alevy, Y., Dickhaus, D.M., Walter, M.J., Holtzman, M.J. (1998) Direct suppression of Stat1 function during adenoviral infection. *Immunity*, **9:** 871-880.

Lowenstein, C.J., Alley, E.W., Raval, P., Snowman, A.M., Snyder, S.H., Russell, S.W., Murphy, W.J. (1993) Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. *Proc.Natl.Acad.Sci.USA.*, **90:** 9730-9743.

Lundberg, J.O., Nordvall, S.L., Weitzberg, E., Kollberg, H., Alving, K. (1996) Exhaled nitric oxide in paediatric asthma and cystic fibrosis. *Arch.Dis.Childhood*, **75:** 323-326.

Luster, A.D. (1998) Chemokines – chemotactic cytokines that mediate inflammation. *New Eng.J.Med.*, **338:** 436-445.

Lyons, C.R. (1995) The role of nitric oxide in inflammation. *Adv.Immunol.*, 60: 323-371.

MacDougall, L.K., Domin, J., Waterfield, M.D. (1995) A family of phosphoinositide 3-kinases in *Drosophilia* identifies a new mediator of signal transduction. *Curr.Opin.Cell Biol.*, **5:** 1404-1415.

MacMacking, J.D., North, R.J., LaCourse, R., Mudgett, J.S., Shah, S.K., Nathan, C.F. (1997) Identification of NOS2 as a protective locus against tuberculosis. *Proc.Natl.Acad.Sci.USA.*, **94:** 5243-5248.

Madrid, L.V., Mayo, M.W., Reuther, J.Y., Baldwin, A.S. (2001) Akt stimulates the transactivation potential of the RelA/p65 subunit of NF-κB through utilization of the IκB kinase and activation of the mitogen-activated protein kinase p38. *J.Biol.Chem.*, **276:** 18934-18940.

Madrid, L.V., Wang, C.Y., Gutteridge, D.C., Schottelius, A.J., Baldwin, A.S., Mayo, M.W. (2000) Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappa B. *Mol.Cell Biol.*, **20:** 1626-1638.

Maehama, T., Dixon, J.E. (1999) PTEN: a tumour suppressor that functions as a phospholipid phosphatase. *Trends Cell Biol.*, **9:** 125-128.

Maiuri, L., Raia, V., de Marco, G. (1997) DNA fragmentation is a feature of cystic fibrosis epithelial cells: a disease with inappropriate apoptosis. *FEBS Lett.*, **408:** 225-231.

Maission, P.P., Inoue, H., Richman-Eisenstat, J.R., Grinberger, D., Jorens, P.G., Housset, B., Pittet, J.F., Wiener-Kronish, J.P., Nadel, J.A. (1994) Novel *Pseudomonas* product stimulates interleukin-8 production in airway epithelial cells *in vitro*. *J.Clin.Invest.*, **93:** 26-32.

Maggi, L.B., Heitmeier, M.R., Scheuner, D., Kaufman, R.J., Buller, R.M.L., Corbett, J.A. (2000) Potential role of PKR in double-stranded RNA induced macrophage activation. *EMBO J.*, **14:** 3630-3638.

Manna, S.K., Aggarwal, B.B. (1998) Interleukin-4 down regulates both forms of tumor necrosis factor receptor and receptor-mediated apoptosis, NF-kappa B, AP-1 and c-jun N-terminal kinase. Comparison with interleukin-13. *J.Biol.Chem.*, **273:** 33333-33341.

Manna, S.K., Aggerwal, B.B. (1998) Alpha-melanocyte-stimulating hormone inhibits the nuclear transcription factor NF-kappa B activation induced by various inflammatory agents. *J.Immunol.*, 161: 2863-2872.

Marini, M., Vittari, E., Hollemborg, J., Mattoli, S. (1992) Expression of the potent inflammatory cytokines, granulocyte-macrophage-colony-stimulating factor, interleukin-6 and interleukin-8 in bronchial epithelial cells of patients with asthma. *J.Allergy Clin.Immunol.*, **89:** 1001-1009.

Marks-Konczalik, J., Chu, S.C., Moss, J. (1998) Cytokine-mediated transcriptional induction of the human inducible nitric oxide synthase gene requires both activator protein 1 and nuclear factor kappaB-binding sites. *J.Biol.Chem.*, **273:** 22201-22208.

Marshall, C.J. (1994) MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr.Opin.Genet.Dev.*, **4:** 82-89.

Martin, E., Ganz, T., Lehrer, R.I. (1995) Defensins and other endogenous peptide antibiotics of vertebrates. *J.Leukoc.Biol.*, **4:** 3-7.

Massengale, A.R.D., Quinn, F., Yankaskas, J., Weissman, D., Maclellan, W.T., Cuff, C., Aranoff, S.C. (1999) Reduced interleukin-8 production by cystic fibrosis airway epithelial cells. *Am.J.Respir.Cell Mol.Biol.*, **20:** 1073-1080.

Mastronarde, J.G., Monick, M.M., Mukaida, N., Matsushima, K., Hunninghake, G.W. (1998) Activator protein-1 is the preferred transcription factor for cooperative interaction with nuclear factor-κB in respiratory syncytial virus-induced interleukin-8 gene expression. *J.Infect.Dis.*, **177:** 1275-1281.

Matsusaka, T., Fujikawa, K., Nishio, Y., Mukaida, N., Matsushima, T., Kishimato, Y., Alcina, S. (1993) Transcription factors NF-IL-6 and NF-κB synergistically activate transcription of the inflammatory cytokines interleukin-6 and interleukin-8. *Proc.Natl.Acad.Sci.USA.*, **90:** 10193-10197.

May, M.J., Ghosh, S. (1997) Rel/NF-kappa B and I kappa B proteins: an overview. *Semin.Cancer Biol.*, **8:** 67-73.

McCall, T.B., Palmer, R.M.J., Moncada, S. (1992) Interleukin-8 inhibits induction of nitric oxide synthase in rat peritoneal neutrophils. *Biochem.Biophys.Res.Commun.*, **186:** 680-685.

McNichols, C.M., Nason, M.W., Guggino, W.B. (1997) A functional CFTR-NBF1 is required for ROMK2-CFTR interaction. *Am.J.Physiol.*, **273:** F843-F848.

Mellott, J.K., Nick, H.S., Waters, M.F., Billiar, T.R., Geller, D.A., Chesrown, S.C. (2001) Cytokine-induced changes in chromatin structure and *in vivo* footprints in the inducible NOS promoter. *Am.J.Physiol.Lung Cell Mol.Physiol.*, **280:** L390-L399.

Meng, Q.H., Springall, D.R., Bishop, A.E. (1998) Lack of inducible nitric oxide synthase in bronchial epithelium: a possible mechanism of susceptibility to infection in cystic fibrosis. *J.Pathol.,* **184:** 323-331.

Milewski, M.I., Michle, J.E., Forrest, J.K., Stafford, D.M., Mayer, B.D., Cheng, J., Guggino, U.B., Stanton, B.A., Cutting, G.R. (2001) A PDZ-binding motif is essential but not sufficient to localise the C terminus of CFTR to the apical membrane. *J.Cell Sci.*, **114:** 719-726.

Misko, T.P., Schilling, R.J., Salvemini, D., Moore, W.M., Currie, M.G. (1993) A fluorometric assay for the measurement of nitrite in biological samples. *Anal.Biochem.*, **214:** 11-16.

Molina Y Vedia, L., McDonald, B., Reep, B., Brine, B., DiSilvo, M., Billiar, T.R., Lapetina, E.G. (1992) Nitric oxide-induced s-nitrosylation of glyceraldehyde 3-phosphate dehydrogenase inhibits enzymatic activity and increased endogenous ADP-ribosylation. *J.Biol.Chem.*, **217:** 24929-24932.

Moncada, S., Higgs, E.A. (1993) Mechanisms of disease – the L-arginine nitric oxide pathway. *N.Engl.J.Med.*, **329:** 2002-2012.

Moncada, S., Palmer, R.M.J., Higgs, E.A. (1991) Nitric oxide: Physiology, pathophysiology and pharmacology. *Pharmacol.Rev.*, **43:** 109-142.

Mori, N., Oishi, K., Sar, B., Mukaida, N., Nagatake, T., Matsushima, K., Yamamoto, N. (1999) Essential role of transcription factor nuclear factor-κB in regulation of

interleukin-8 gene expression by nitrite reductase from *Pseudomonas aeruginosa* in respiratory epithelial cells. *Infect.Immun.*, **67:** 3872-3878.

Morris, S.M., Billia, T.R. (1994) New insights into the regulation of inducible nitric oxide synthase. *Am.J.Physiol.*, **266:** E829-E839.

Moser, C., Kjaergaard, S., Pressler, T., Kharazmi, A., Koch, C., Hoiby, N. (2000) The immune response to chronic *Pseudomonas aeruginosa* lung infection in cystic fibrosis patients is predominantly of the Th2 type. *APMIS.,* **108:** 335-329.

Moyer, B.D., Denton, J., Karlson, K.H., Reynolds, D., Wang, S.S., Michle, J.E., Milewski, M., Cutting, G.R., Guggino, W.B., Li, M., Stanton, B.A. (1999) A PDZ-interacting domain in CFTR is an apical membrane polarization signal. *J.Clin.Invest.*, **104:** 1353-1361.

Moyer, B.D., Dunhaime, M., Shaw, C., Denton, J., Reynolds, D., Karlson, K.H., Pfeiffer, J., Wang, S.S., Michle, J,E., Milewski, M., Cutting, G.R., Guggino, W.B., Li, M., Stanton, B.A. (2000) The PDZ-interacting domain of cystic fibrosis transmembrane conductance regulation is required for functional expression in the apical plasma membrane. *J.Biol.Chem.*, **275:** 27069-27074.

Muhl, H., Pfeilschifter, J. (1995) Amplification of nitric oxide synthase expression by nitric oxide in interleukin-1 stimulated rat mesangial cells. *J.Clin.Invest.*, **95:** 1941-1946.

Muhlebach. M.S., Stewart, P.W., Leigh, M.W., Noah, T.L. (1999) Quantitation of inflammatory responses to bacteria in young cystic fibrosis and control patients. *Am.J.Respir.Crit.Care Med.*, **160:** 186-191.

Mukaida, N., Mahe, Y., Matsushima, K. (1990) Cooperative interaction of nuclear factor-κB and cis-regulatory enhancer binding protein-like factor binding elements in activating the interleukin-8 gene by proinflammatory cytokines. *J.Biol.Chem.*, **265:** 21128-21132.

Mukaida, N., Okamota, S., Ishihawa, Y., Matsushima, K. (1994) Molecular mechanism of interleukin-8 gene expression. *J.Leukoc.Biol.*, **56:** 554-558.

Murphy, P.M. (1994) The molecular biology of leucocyte chemoattractant receptors. *Annu.Rev.Immunol.*, **12:** 593-633.

Nathan, C. (1997) Inducible nitric oxide synthase: what difference does it make? *J.Clin.Invest.*, **100:** 2417-2423.

Neithommer, M., Kim, E., Sheng, M. (1996) Interaction between the C-terminus of NMDA receptor subunits and multiple members of the PSD-95 family of membrane-associated guanylate kinases. *J.Neurosci.*, **16:** 2157-2163.

Nicholson, S., Bonecini-Almeida, M.D.G., Lapa e Silva, J.R., Nathan, C., Xie, Q-W, Mimford, R., Weidner, J.R., Calaycay, J., Geng, J., Boechat, N. (1996) Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *J.Exp.Med.*, **183:** 2293-2302.

Noah, T.L., Black, H.R., Cheng, P.W., Wood, R.E., Leigh, M.W. (1997) Nasal and bronchoalveolar lavage fluid cytokines in early cystic fibrosis. *J.Infect.Dis.,* **175:** 638-647.

Norris, J.L., Baldwin, A.S. (1999) Oncogenic Ras enhances NF-kappa B transcriptional activity through Raf-dependent and Raf-independent activated protein kinase signalling pathways. *J.Biol.Chem.*, **274:** 13841-13846.

Nunokawa, Y., Ishida, N., Tanaka, S. (1994) Promoter analysis of human inducible nitric oxide synthase gene associated with cardio-vascular homeostasis. *Biochem.Biophys.Res.Commun.*, **200:** 802-807.

Oliveira, I.C., Mukaida, N., Matsushima, K., Vilcek, J. (1994) Transcriptional inhibition of the interleukin-8 gene by interferon is mediated by the NF-κB site. *Mol.Cell Biol.*, **14:** 5300-5308.

Oppenheim, J.J., Zachariae, C.O.C. Muikada, N., Matsushima, K. (1991) Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu.Rev.Immunol.*, **9:** 617-648.

Oswald, I.P., Gazzinelli, R.T., Sher, A., James, S.L. (1992) IL-10 synergizes with IL-4 and transforming growth factor beta to inhibit macrophage cytotoxic activity. *J.Immunol.*, **148:** 3578-3582.

Ozes, O.N., Mayo, L.D., Gustin, J.A., Pfeffer, S.R., Pfeffer, L.M., Donner, D.B. (1999) NF-kappa B activation by tumor necrosis factor requires the Akt serine-threonine kinase. *Nature*, **401:** 82-85.

Pacelli, R., Wink, D.A., Cook, J.A., Krishna, M.C., DeGraff, W., Freidman, N., Tsokos, M., Samuni, A., Mitchell, J.B. (1995) Nitric oxide potentiates hydrogen peroxide-induced killing of *Escherichia coli. J.Exp.Med.*, **182:** 1469-1479.

Pap, M., Cooper, G.M. (1998) Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-kinase / Akt cell survival pathway. *J.Biol.Chem.*, **273:** 19929-19932.

Park, Y.C., Lee, C.H., Kong, H.S., Chung, H.T., Kim, H.D. (1997) Wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase, enhances LPS-induced NO production from murine peritoneal macrophages. *Biochem.Biophys.Res.Commun.*, **240:** 692-696.

Pawson, T., Scott, J.D. (1997) Signalling through scaffold, anchoring and adaptor proteins. *Science*, **278:** 2075-2080.

Perez, A., Risma, K.A., Eckman, E.A., Davis, P.B. (1996) Overexpression of R domain Eliminates cAMP-stimulated Cl^- secretion in 9HTEo$^-$ cells in culture. *Am.J.Physiol.*, **217:** L85-L92.

Pfeilschifter, J. (1991) Anti-inflammatory steroids inhibit cytokine induction of nitric oxide synthase in rat renal mesangial cells. *Eur.J.Pharmacol.*, **195:** 179-180.

Pier, G.B., Grout, M., Zaidi, T.S., Goldberg, J.B. (1996) How mutant CFTR may contribute to *Pseudomonas aeruginosa* infection in cystic fibrosis. *Am.J.Respir.Crit.Care Med.*, **154:** 5175-5182.

Plyte, S.E., Hughes, K., Nikolakaki, E., Pulverer, B.J., Woodgett, J.R. (1992) Glycogen synthase kinase-3: functions in oncogenesis and development. *Biochim.Biophys.Acta.*, **114:** 147-162.

Premack, B.A., Schall, T.J. (1996) Chemokine receptors, gateways to inflammation and infection. *Nat.Med.*, **2:** 1174-1178.

Quie, P.G. (1986) Lung defence against infection. *J.Pediatr.*, **108:** 813-816.

Quinton, P.M. (1983) Chloride impermeability in cystic fibrosis. *Nature*, **301:** 421-422.

Radomski, M.W., Palmer, R.M., Moncada, S. (1990) Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc.Natl.Acad.Sci.USA.*, **87:** 10043-

Raghuram, V., Mak, D.D., Fosket, J.K. (2001) Regulation of cystic fibrosis transmembrane conductance regulator single-channel gating by bivalent PDZ-domain-mediated interaction. *Proc.Natl.Acad.Sci.USA.*, **98:** 1300-1305.

Rameh, L.E., Cantley, L.C. (1999) The role of phosphoinositide 3-kinase lipid products in cell function. *J.Biol.Chem.*, **274:** 8647-8350.

Ramsey, B.W., Wentz, W.R., Smith, A.L., Richardson, M., Williams-Warren, J., Dedges, D.J., Gibson, R., Redding, G.J., Lent, K., Harris, K. (1991) Predictive value of oropharyngeal cultures for identifying lower airway bacteria in cystic fibrosis patients. *Am.Rev.Respir.Dis.*, **144:** 331-333.

Ratner, A.J., Bryan, R., Weber, A., Nguyen, S., Barnes, D., Pitt, A., Gelber, S., Cheung, A., Prince, A. (2001) Cystic fibrosis pathogens activate Ca^{2+}-dependent mitogen-activated protein kinase signalling pathways in airway epithelial cells. *J.Biol.Chem.*, **276:** 19267-19275.

Reddel, R.R., Ke, Y., Gerwin, B.I., McMenamin, M.G., Lechner, J.F., Su, R.T., Brash, D.E., Park, J.B., Rhim, J.S., Harris, C.C. (1988) Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. *Caner Res.*, **48:** 1904-1909.

Reddy, S.A.G., Huang, J.H., Liao, W.S.L. (1997) Phosphatidylinositol 3-kinase in interleukin-1 signalling: a physical interaction with the interleukin-1 receptor and requirement in NF-kappaB and AP-1 activation. *J.Biol.Chem.*, **272:** 29167-29173.

Reddy, S.A.G., Huang, J.H., Liao, W.S.L. (2000) Phosphatidylinositol 3-kinase as a mediator of TNF-induced NF-kappaB activation. *J.Immunol.*, **164:** 1355-1363.

Reinecker, H-C., Loy, E.Y., Ringler, D.J., Mchta, A., Rombeau, J.L. MacDermott, R.P. (1995) Monocyte-chemoattractant protein-1 gene expression in intestinal epithelial cells and inflammatory bowel disease mucosa. *Gastroenterology*, **108:** 40-50.

Rene, A., Massengale, D., Quinn, F. (1999) Reduced interleukin-8 production by cystic fibrosis airway epithelial cells. *Am.J.Respir.Cell Mol.Biol.*, **20:** 1073-1080.

Rennard, S.I. (1994) Airway epithelial cells: functional roles in airway disease. *Am.J.Respir.Crit.Care Med.*, **150:** S27-S30.

Rich, D.P., Berger, H.A., Cheng, S.H. (1993) Regulation of the cystic fibrosis transmembrane conductance regulator chloride channel by negative charge in the R domain. *J.Biol.Chem.*, **268:** 20259-20267.

Richmann-Eisanstat, J.B.Y., Jorens, P.G., Herbert, C.A., Ueki, I., Nadel, J.A. (1993) Interleukin-8: an important chemoattractant in sputum of patients with chronic inflammatory airway diseases. *Am.J.Physiol.*, **264:** L413-L418.

Riordan, J.R., Rommens, J.M., Kerem, B.S., Alon, N., Rozmahel, R., Grzelczak, A., Zielenski, J., Lok, S., Plavsic, N., Chou, J.L., Drumm, M.L., Iannuzzi, M.C., Collins, F.S., Tsui, L.C. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science*, **245:** 1066-1073.

Robbins, R.A., Barnes, P.J., Springall, D.R., Warren, J.B., Kwon, O.J., Buttery, L.D., Wilson, A.J., Geller, D.A., Polak, J.M. (1994) Expression of inducible nitric oxide in human lung epithelial cells. *Biochem.Biophys.Res.Comm.*, **203:** 209-218.

Robbins, R.A., Springall, D.R., Warren, J.B., Kwon, O.J., Buttery, L.D., Wilson, A.J., Adcock, I.M., Riveros, M.V., Moncada, S., Polak, J.M., Barnes, P.J. (1994)

Inducible nitric oxide synthase is increased in murine lung epithelial cells by cytokine stimulation. *Biochem.Biophys.Res.Commun.*, **198:** 835-943.

Robinson, M.J., Cobb, M.H. (1997) Mitogen-activated protein kinase pathways. *Curr.Opin.Cell Biol.*, **9:** 180-186.

Rollins, B.J. (1997) Chemokines, review article. *Blood*, **90:** 909-928.

Romashkova, J.A., Makarov, S.S. (1999) NF-kappa B is a target of Akt in anti-apoptotic signalling. *Nature*, **401:** 86-90.

Rosbe, K.W., Mims, J.W., Prazma, J., Petrusz, P., Rose, A., Drake, A.F. (1996) Immunohistochemical localisation of nitric oxide synthase activity in upper respiratory epithelium. *Laryngoscope*, **106:** 1075-1079.

Rosenweig, A., Seidman, C.E. (1991) Atrial natriuetic factor and related peptide hormones. *Annu.Rev.Biochem.*, **60:** 14636-14642.

Ruef, C., Jefferson, D.M., Schlegel-Hauter, S.E., Suter, S. (1993) Regulation of cytokine secretion by cystic fibrosis airway epithelial cells. *Eur.Respir.J.*, **6:** 1429-1436.

Russell, J.P., Diamond, G., Tarver, A.P., Scanlin, T.F., Bevins, C.L. (1996) Coordinate induction of two antibiotic genes in tracheal epithelial cells exposed to the inflammatory mediators lipopolysaccharide and tumour necrosis factor alpha. *Infect.Immun.*, **64:** 1565-1568.

Saiman, L., Prince, A. (1993) *Pseudomonas aeruginosa* pili bind to asiolo GM_1 which is increased on the surface of cystic fibrosis epithelial cells. *J.Clin.Invest.*, **92:** 1875-1880.

Saura, M., Zanagoza, C., Bao, C., McMillan, A., Lowenstein, C.J. (1999) Interaction of interferon regulatory factor-1 and nuclear factor-κB during activation of inducible nitric oxide synthase transcription. *J.Mol.Biol.*, **289:** 459-471.

Schall, T.J., Bacon, K., Tay, K.J., Goeddel, D.V. (1990) Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature*, **347:** 669-671.

Schuster, A., Haarman, A., Wahn, V. (1995) Cytokines in neutrophil-dominated airway inflammation inpatients with cystic fibrosis. *Eur.Arch.Otorhinolaryngol.Suppl.*, **1:** S59-S60.

Schwiebert, E.M., Morales, M.M., Devidas, S., Egan, M.E., Guggino, W.B. (1998) Chloride channel and chloride conductance regulator domains of CFTR, the cystic fibrosis transmembrane conductance regulator. *Proc.Natl.Acad.Sci.USA.*, **95:** 2674-2679.

Schwiebert, L.M., Estell, K., Propst, S.M. (1999) Chemokine expression in CF epithelia: implications for the role of CFTR in RANTES expression. *Am.J.Physiol.*, **276:** C700-C710.

Sen, R., Baltimore, D. (1986) Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell*, **46:** 705-716.

Shaw, M., Cohen, P., Alessi, D.R. (1998) The activation of protein kinase B by H_2O_2 or heat shock is mediated by phosphoinositide 3-kinase and not by mitogen-activated protein kinase-activated protein kinase-2. *Biochem.J.*, **336:** 241-246.

Shoumacher, R.A., Shoemaker, R.L., Halm, D.R., Tallant, E.A., Wallace, R.W., Frizzell, R.A. (1987) Phosphorylation fails to activate chloride channels from cystic fibrosis airway cells. *Nature*, **330:** 752-758.

Siebenlist, U., Franzoso, G., Brown, K. (1994) Strucure, regulation and function of NF-κB. *Annu.Rev.Cell Biol.*, **10:** 405-455.

Singh, S.P., Schaefer, A.L., Parsek, M.P., Moninger, T.O., Welsh, M.J., Greenberg, E.P. (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature*, **407:** 762-764.

Sironi, M., Sciacca, F.L., Matteuci, C., Conni, M., Vecchi, A., Bernasconi, S., Minty, A., Caput, D., Ferrara, P., Colotta, F., Montovani, A. (1994) Regulation of endothelial and mesothial cell function by interleukin-13: selective induction of vascular cell adhesion molecule-1 and amplification of interleukin-6 production. *Blood*, **84:** 1913-1921.

Sizemore, N., Leung, S., Stork, G.R. (1999) Activation of Phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappa B p65/Rel A subunit. *Mol.Cell Biol.*, **19:** 4798-4805.

Smith, A.W., Green, J., Eden, C.E., Watson, M.L. (1999) Nitric oxide-induced potentiation of the killing of *Burkholderia cepacia* by reactive oxygen species: implications for cystic fibrosis. *J.Med.Microbiol.*, **48:** 419-423.

Smith, J.J., Travis, S.M., Greenberg, E.P., Welsh, M.J. (1996) Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell*, **85:** 229-236.

Smith, R.S., Fedyk, E.R., Springer, T.A., Mukaida, N., Iglewski, B.H., Phipps, R.P. (2001) IL-8 production in human lung fibroblasts and epithelial cells activated by the *Pseudomonas* autoinducer N-3-oxododenconoyl homoserine lactone is transcriptionally regulated by NF-κB and activator protein-2. *J.Immunol.*, **167:** 366-374.

Sonada, Y., Wantanabe, S., Matsumoto, Y., Aizu-Yokota, E., Kasahara, T. (1999) FAK is the upstream signal protein of the phosphatidylinositol 3-kinase-Akt survival pathway in hydrogen peroxide-induced apoptosis of a human glioblastoma cell line. *J.Biol.Chem.*, **274:** 10566-10570.

Stambolic, V., Suzuli, A., de la Pompa, J.L., Brothers, G.M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J.M., Siderovski, D.P. (1998) Negative regulation of PKB/Akt dependent cell survival by the tumour suppressor PTEN. *Cell*, **95:** 29-39.

Steagall, W,K., Elmer, H.L., Brady, K.G., Kelley, T.J. (2000) Cystic fibrosis transmembrane conductance regulator-dependent regulation of epithelial inducible nitric oxide synthase expression. *Am.J.Respir.Cell Mol.Biol.*, **22:** 45-50.

Stecenko, A., Breyer, R., Canonico, A., Dworski, R., Lung, G., Torri, K., Brigham, K. (1997) Exaggerated inflammatory response in CF airway epithelial cells (Abstract). *Pediatric Pulmonol.*, **S14:** 320.

Steck, P.A., Pershouse, M.A., Jasser, S.A., Yung, W.K., Lin, H., Ligon, A.H., Langford, L.A., Baugmord, M.L., Hattier, T., Davis, T. (1997) Identification of a candidate tumour suppression gene MMAC1, at chromosome 10q23.3 that is mutated in multiple advance cancers. *Nat.Genet.*, 15: 356-362.

Stefano, G.B. (1998) Antagonism of LPS and IFN-gamma induction of iNOS in human sapherous vein endothelium by morphine and anadamide by nitric oxide inhibition of adenylate cyclase. *J.Cardiovasc.Pharmacol.*, **32:** 813-

Stefano, G.B., Magazine, H.I. (2001) Nitric oxide autoregulation and its significance. *Mod.Asp.Immunobiol.*, **1:** 182-186.

Stein, R.C., Waterfield, M,D. (2000) PI3-kinase inhibition: a target for drug development? *Mol.Med.Today*, **6:** 347-357.

Stenger, S., Donhauser, N., Thuring, H., Rollinghoff, M., Bogdan, C. (1996) Reactivation of latent leishmaniasis by inhibition of inducible nitric oxide synthase. *J.Exp.Med.*, **183:** 1501-1514.

Stephens, L., Anderson, K., Stokoe, D., Erdjument-Bromage, H., Painter, G.F., Holmes, A.B., Gaffney, P.R., Reese, C.B., MaCormick, F., Tempst, P., Coadwell, J., Hawkins, P.T. (1998) Protein kinase B kinases that mediate phosphatidylinositol-3,4,5-trisphosphate-dependent activation of protein kinase B. *Science*, **279:** 710-714.

Stephens, L.R., Jackson, T.R., Hawkins, P.T. (1993) Agonist-stimulated synthesis of phosphatidylinositol-3,4,5-trisphosphate: a new intracellular signalling system? *Biochim.Biophys.Acta.*, **1179:** 27-75.

Stuehr, D.J., Griffith, O.W. (1992) Mammalian nitric oxide synthases. *Adv.Enzym.*, **65:** 287-346.

Stutts, M.J., Cotton, C.U., Yankaskas, J.R., Knowles, M.R., Gatzy, J.T., Boucher, R.C. (1985) Chloride uptake into cultured airway epithelial cells from cystic fibrosis patients and normal individuals. *Proc.Natl.Acad.Sci.USA.*, **82:** 6677-6681.

Sutherland, C., Leighton, I.A., Cohen, P. (1993) Inactivation of glycogen synthase kinase-3 beta by phosphorylation: new kinase connections in insulin and growth factor signalling. *Biochem.J.*, **296:** 15-19.

Suyang, H., Phillips, R., Douglas, I., Ghosh, S. (1996) Role of unphosphorylated, newly synthesised IκBβ in persistent activation of NF-κB. *Mol.Cell Biol.*, **16:** 5444-5449.

Szabo, C., Cuzzocrea, S., Zingarelli, B., O'Connor, M., Salzman, A.L. (1997) Endothelial dysfunction in a rat model of endotoxic shock: importance of the activation of poly(ADP-ribose) synthase by peroxynitrite. *J.Clin.Invest.*, **100:** 723-735.

Tabary, O., Escotte, S., Couteil, J.P., Hubert, D., Dusser, D., Puchelle, E., Jacquot, J. (1999) Genistein inhibits constitutive and inducible NF-κB activation and decreases IL-8 production by human cystic fibrosis bronchial gland cells. *Am.J.Pathol.*, **155:** 473-481.

Tabary, O., Escotte, S., Couteil, J.P., Hubert, D., Dusser, D., Puchelle, E., Jacquot, J. (2000) High susceptibility for cystic fibrosis human airway gland cells to produce IL-8 through the IκB kinase α pathway in response to extracellular NaCl content. *J.Immunol.*, **164:** 3377-3384.

Taylor, B.S., DeVera, M.E., Ganster, R.W., Wang, Q., Shapiro, R.A., Morris, S.M., Billiar, T.R., Geller, D.A. (1998) Multiple NF-κB enhancer elements regulate cytokine induction of the human inducible nitric oxide synthase gene. *J.Biol.Chem.*, **273:** 15148-15156.

Thompson, J.E., Phillips, R.J., Erdjument-Bromage, H., Tempst, P., Ghosh, S. (1995) IκBβ regulates the persistent response in a biphasic activation of NF-κB. *Cell*, **80:** 573-582.

Tirouvanziam, R., de Bentzmann, S., Hubeau, C. (2000) Inflammation and infection in naïve human cystic fibrosis airway grafts. *Am.J.Respir.Cell Mol.Biol.*, **23:** 121-127.

Titheradge, M.A. (1999) Nitric oxide in septic shock. *Biochim.Biophys.Acta.*, **1411:** 437-455.

Toker, A. (2000) Protein kinases as mediators of phosphoinositide 3-kinase signalling. *Mol.Pharmacol.*, **57:** 652-658.

Travis, S.M., Conway, B.A., Zabner, J., Smith, J.J., Amderson, N.N., singh, P.K., Greenberg, E.P., Welsh, M.J. (1999) Activity of abundant antimicrobials in the human airway. *Am.J.Respir.Cell Mol.Biol.*, **20:** 872-879.

Tummler, B., Kiewitz, C. (1999) Cystic fibrosis: an inherited susceptibility to bacterial respiratory infections. *Mol.Med.Today*, **5:** 351-358.

Uetani, K., Arroliga, M.E., Erzurum, S.C. (2001) Double-stranded RNA dependence of nitric oxide synthase 2 expression in human bronchial epithelial cell lines BET-1A and BEAS-2B. *Am.J.Respir.Cell Mol.Biol.*, **24:** 720-726.

Uetani, K., Der, S.D., Zamanian-Daryoush, M., De la Motte, C., Lieberman, B.Y., Williams, B.R.G., Erzurum, S.C. (2000) Central role of double-stranded RNA-activated protein kinase in microbial induction of nitric oxide synthase. *J.Immunol.*, **165:** 92-100.

Van Dedden, C., Iglewski, B.H. (1998) Cell-to-cell signalling and *Pseudomonas aeruginosa* infections. *Emerg.Infect.Dis.*, 4: 551-560.

Vanhaesbroeck, B., Leevers, S.J., Panaytou, G., Waterfield, M.D. (1997) Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem.Sc.*, **22:** 267-272.

Vanhaesbroeck, B., Waterfield, M.D. (1999) Signalling by distinct classes of phosphoinositide 3-kinases. *Experi.Cell Res.*, **253:** 239-254.

Van Heeckeren, A., Walenga, R., Konstan, M.W., Bonfield, T., Davis, P.B., Ferkol, T. (1997) Excessive inflammatory response of cystic fibrosis mice to bronchopulmonary infection with *Pseudomonas aeruginosa. J.Clin.Invest.*, **100:** 2810-2815.

Vankeerberghen, A., Cuppens, H., Cassiman, J. (2001) The cystic fibrosis transmembrane conductance regulator: an intriguing protein with plieotropic function. *J.Cystic Fibrosis*, **1:** 13-19.

Vazquez, F., Grossman, S.R., Takahashi, Y., Rokas, M.V., Nakamura, N., Sellers, W.R. (2001) Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex. *J.Biol.Chem.*, **276:** 48627-48630.

Vazquez, F., Sellers, W.R. (2000) The PTEN tumor suppressor protein: an antagonist of phosphoinositide 3-kinase signalling. *Biochim.Biophys.Acta.*, **1470:** M21-M35.

Venkatakrishnan, A., Stecenko, A., King, G., Blackwell, T.R., Brigham, K., Christman, J.W., Blackwell, T.S. (2000) Exaggerated activation of nuclear factor-κB and altered IκBβ processing in cystic fibrosis bronchial epithelial cells. *Am.J.Respir.Cell Mol.Biol.*, **23:** 396-403.

Vlahos, C.J., Mattr, W.F., Hui, K.Y., Brown, R.F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J.Biol.Chem.*, **269:** 5241-5248.

Walker, G., Pfeilschifter, J., Kunz, D. (1997) Mechanisms of suppression of inducible nitric-oxide synthase (iNOS) expression in interferon (IFN)-gamma-stimulated RAW 264.7 cells by dexamethasone: evidence for glucocorticoid-induced degradation of iNOS protein by calpain as a key step in post-transcriptional regulation. *J.Biol.Chem.*, **272:** 16679-16687.

Wang, D., Baldwin, A.S. (1998) Activation of nuclear factor-kappa B-dependent transcription by tumor necrosis factor-alpha is mediated through phosphorylation of RelA/p65 on serine 529. *J.Biol.Chem.*, **273:** 29411-29416.

Wang, S., Yue, H., Derin, R.B., Guggino, W.B., Li, M. (2000) Accessory protein facilitated CFTR-CFTR interaction, a molecular mechanism to potentiate the chloride channel activity. *Cell*, **103:** 169-179.

Wang, Q.D., Kim, S.H., Wang, X.F., Evers, B.M. (2000) Activation of NF-κB binding in HT-29 colon cancer cells by inhibition of phosphatidylinositol 3-kinase. *Biochem.Biophys.Res.Commun.*, **273:** 853-858.

Watkins, D.N., Peroni, D.J., Basclain, K.A., Garlepp, M.J., Thompson, P.J. (1997) Expression and activity of nitric oxide synthases in human airway epithelium. *Am.J.Respir.Cell Mol.Biol.*, **16:** 629-639.

Watnick, P., Kolter, R. (2000) Biofilm, city of microbes. *J.Bacteriol.*, **182:** 2675-2679.

Watson, M.L. (2002) Chemokines – linking receptors to response. *Immunology*, **105:** 121-124.

Weber, A.J., Soong, G., Bryan, R., Shahryar, S., Prince, A. (2001) Activation of NF-κB in airway epithelial cells is dependent on CFTR trafficking and Cl^- channel function. *Am.J.Physiol.Lung Cell Mol.Physiol.*, **281:** L71-L78.

Wei, X-Q., Charles, I.G., Smith, A., Ure, J., Fend, G-J., Huang, F-P., Xu, D., Muller, W., Moncada, S., Liew, F.Y. (1995) Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature*, **375:** 408-411.

Welch, G.N., Upchurch, G.R., Loscalzo, J. (1995) Nitric oxide as a vascular modulator. *Blood Rev.*, **9:** 262-269.

Wells, T.N.C., Power, C.A., Proudfoot, A.E.I. (1998) Definition, function and pathophysiological significance of chemokine receptors. *TIPS.*, **19:** 376-380.

Welsh, M.J. (1986) An apical membrane chloride channel in human tracheal epithelium. *Science*, **232:** 1648-1650.

Welsh, M.J., Smith, A.E. (1993) Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. *Cell*, **73:** 1251-1254.

Wesselborg, S., Bauer, M.K.A., Vongt, M., Schmitz, M.L., Schulze-Osthoff, K. (1997) Activation of transcription factor NF-kappa B and p38 mitogen-activated protein kinase is mediated by distinct and separate stress effector pathways. *J.Biol.Chem.*, **272:** 12422-12429.

Widdicombe, J.H. (2000) Yet another role for the cystic fibrosis transmembrane conductance regulator. *Am.J.Respir.Cell Mol.Biol.*, **22:** 11-14.

Widdicombe, J.H., Welsh, M.J., Finkbeiner, W.E. (1985) Cystic fibrosis decreases the apical membrane permeability of monolayers cultured from cells of tracheal epithelium. *Proc.Natl.Acad.Sci.USA.*, **82:** 6167-6171.

Wilmott, R.W., Kaseb, J., Killian, P.L., Benjamin, W.R., Douglas, S.D., Wood, R.E. (1990) Increased levels of interleukin-1 in bronchoalveolar lavage washings from children with bacterial pulmonary infections. *Am.Rev.Respir.Dis.*, **142:** 365-368.

Wine, J.J. (1999) The genesis of cystic fibrosis lung disease. *J.Clin.Invest.*, **103:** 309-312.

Winter, M.C., Welsh, M.J. (1997) Stimulation of CFTR activity by its phosphorylated R domain. *Nature*, **389:** 294-296.

Woodgett, J.R. (1991) cDNA cloning and properties of glycogen synthase kinase-3. *Methods Enzymol.*, **200:** 564-577.

Woodgett, J.R. (1991) A common denominator linking glycogen metabolism, nuclear oncogenes and development. *TIBS.*, **16:** 177-181.

Wright, K., Kolios, G., Westwick, J., Ward, S.G. (1999) Cytokine-induced apoptosis in epithelial HT-29 cells is independent of nitric oxide formation. Evidence for an interleukin-13 driven phosphatidylinositol 3-kinase dependent survival mechanism. *J.Biol.Chem.*, **274:** 17193-17201.

Wright, K.L., Ward, S.G. (2000) Interactions between phosphatidylinositol 3-kinase and nitric oxide: explaining the paradox. *Mol.Cell Biol.Res.Commun.*, **4:** 137-143.

Wright, K., Ward, S.G., Kolios, G., Westwick, J. (1997) Activation of phosphatidylinositol 3-kinase by interleukin-13. An inhibitory signal for inducible nitric oxide synthase expression in epithelial cell line HT-29. *J.Biol.Chem.*, **272:** 12626-12633.

Wu, X., Hepner, K., Castelino-Prabhu, S., Do, D., Kaye, M.B., Yuan, X.J., Wood, J., Ross, C., Sawyers, C.L., Whang, Y.E. (2000) Evidence for regulation of the PTEN tumour suppressor by a membrane-localised multi-PDZ domain containing scaffold protein MAGI-2. *Proc.Natl.Acad.Sci.USA.*, **97:** 4233-4238.

Wymann, M.P., Bulgarelli-Leva, G., Zvelebil, M.J., Pirola, L., Vanhaesebroeck, M., Waterfield, M.D., Panayotou, G. (1996) Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of lys-802, a residue involved in the phosphate transfer reaction. *Mol.Cell Biol.*, **16:** 1722-1733.

Wymann, M.P., Pirola, L. (1998) Structure and function of phosphoinositide 3-kinases. *Biochem.Biophys.Acta.*, **1436:** 127-150.

Xie, Q.W., Cho, H.J., Calaycay, J., Mumford, R.A., Swiderek, K.M., Lee, T.D., Ding, A., Troso, T., Nathan, C. (1992) Cloning and characterisation of inducible nitric oxide synthase from mouse macrophages. *Science*, **256:** 225-228.

Xu, K.D., McFeter, G.A., Stewart, P.S. (2000) Biofilm resistance to antimicrobial agents. *Microbiology*, **146:** 2481-2493.

Yankaskas, J.R., Cottonm C.U., Knowles, M.R., Gatzy, J.T., Boucher, R.C. (1985) [a] Culture of human nasal epithelial cells on collagen matrix supports: a comparison of bioelectric properties of normal and cystic fibrosis epithelia. *Am.Rev.Respir.Dis.*, **132:** 1281-1287.

Yankaskas, J.R., Gatzy, J.T., Knowles, M.R., Boucher, R.C. (1985) [b] Persistence of abnormal chloride ion permeability in cystic fibrosis nasal epithelial cells in heterogeneous culture. *Lancet*, **1:** 954-956.

Yasumoto, K., Okamoto, S.I., Mukaida, N., Murakami, S., Mai, M., Matsushima, K. (1992) Tumour necrosis factor α and interferon γ synergiatically induce interleukin-8 production in a human gastric cancer cell line through acting concurrently on AP-1 and NF-κB-like binding sites of the interleukin-8 gene. *J.Biol.Chem.*, **267:** 22506-22511.

Zabner, J., Smith, J.J., Karp, P.H., Widdicombe, J.H., Welsh, M.J. (1998) Loss of CFTR chloride channels alters salt absorption by cystic fibrosis airway epithelia *in vitro*. *Mol.Cell Biol.*, **2:** 397-403.

Zar, H., Saiman, L., Quittell, L., Prince, A. (1995) Binding of *Pseudomonas aeruginosa* to respiratory epithelial cells from patients with various mutations in the cystic fibrosis transmembrane conductance regulator. *J.Pediatr.*, **126:** 230-233.

Zeitlin, P.L. (1999) Novel pharmacologic therapies for cystic fibrosis. *J.Clin.Invest.*, **103:** 447-452.

Zhang, P., Summer, W.R., Bagby, G.J., Nelson, S. (2000) Innate immunity and pulmonary host defence. *Immunol.Rev.*, **173:** 39-51.

Zurawski, G., DeVries, J.E. (1994) Interleukin-13, an interleukin-4-like cytokine that acts on monocytes and B cells, but not on T cells. *Immunol.Today*, **15:** 19-26.